CHAPTER 5

Developmental effects of cannabinoids on zebrafish larvae

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Abstract

Here, we examine the effects of the cannabinoids Δ^9-THC, (R)-(+)-[2,3-Dihydro-5-methyl- 3-(4-morpholinylmethyl)pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]- 1-napthalenylmethanone (WIN 55,212-2) and 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol (CP 55,940), and the cannabinoid antagonist (AM251). Exposures were either acute (1-12 h exposure at 108 hour post fertilization [hpf]); or chronic (96 h exposure starting at 24 hpf). Geometric range-finding was used to determine the experimental concentrations. LC_{50} was determined based on mortality at 5 days post fertilisation (dpf). At day 5, behavioural analysis (visual motor response test) was carried out in which movement of individual larvae was analysed using automated video-tracking. With acute exposure, embryos showed a biphasic response to the dark challenge with all three cannabinoids tested. This response consisted of stimulation of locomotor activity at low concentrations, suppression at high doses. With chronic exposure, embryos habituated to the effects of all three cannabinoids when assayed with the dark challenge phase. Furthermore, the excitation was ameliorated when the antagonist AM251 was co-administered with the cannabinoid. When AM251 was administered on its own (chronically or acutely), the locomotor activity was suppressed at high concentrations. We examined the embryos for a range of malformations after chronic exposure to cannabinoid. Only Δ^9-THC was associated with a significant increase in malformations at 5d (yolk sac and pericardial oedema, bent tail). We conclude that cannabinoids have behavioural effects in zebrafish that are comparable to some of those reported in the literature for mammals. In particular, the acute exposure response resembles behavioural effects reported for adult rodents. Our data are consistent with these behavioural effects being mediated, at least in part, by the CB_1 receptor.

Introduction

Zebrafish embryos have great promise for use in high-throughput screening of new drug candidates (Bull and Levin 2000, Lieschke and Currie 2007, Ali et al. 2011, Ali et al. 2011a, Ali et al. 2011b, Ali et al. 2011c). The zebrafish model is not an alternative to rodent models in drug screening, but is complementary to them (Ali et al. 2011). It could be helpful in studies demanding rapid, high-throughput and low-cost assays, such as in the early (pre-regulatory) stages of drug testing (Teraoka et al. 2003, Redfern et al. 2008) and also for behavioral testing (Best et al. 2008, Champagne et al. 2010, Rihel et al. 2010, Ali et al. 2011a). Many basic cellular and molecular pathways regulated by different compounds, and by stress stimuli, are similar between the zebrafish and mammals (Voelker et al. 2007, Schaaf et al. 2008).

Purification and structural elucidation of Δ^9-THC (Gaoni and Mechoulam 1964) has led to the discovery of many pharmacological properties of cannabinoids. Δ^9-THC and its derivatives are being studied for their psychotropic properties and other pharmacological activities, including their possible actions as anticonvulsants, antidepressants, hypotensives, bronchodilators, analgesics and the ability to lower intraocular pressure (Holdcroft et al. 2006). Cannabinoids have also been examined for suitability in the symptomatic treatment of multiple sclerosis (Zajicek et al. 2005, Baker et al. 2007). Unfortunately, cannabinoids may have serious, undesirable effects such as dependency, a possible causative association with psychotic illness, and cognitive impairment including deleterious effects on memory (Niyuhire et al. 2007, Hoffman et al. 2007, Morgan et al. 2009, Cooper and Haney 2009, Justinova et al. 2009).

Rodent models have been used to explore the teratological, toxicological and behavioural effects of cannabinoids and their receptor agonists (Sulcova et al.

1998, Norwood et al. 2003, Drews et al. 2005, Wiley et al. 2007). Cannabinoid receptor type 1 (CB_1) (Lolait et al. 1990), and Cannabinoid receptor type 2 (CB_2) (Munro et al. 1993) are G-protein-coupled receptors (Pertwee 2008). Extensive work has been done to understand their role (Rodriguez-Martin et al. 2007, Braida et al. 2007, Migliarini and Carnevali 2009). Several previous studies showed that the behavioral effect of Δ^9-THC is mediated by the central CB_1 receptor in rats (Tseng and Craft 2004). CB_1 (Lam et al. 2006) and CB_2 (Rodriguez-Martin et al. 2007) receptors have also been reported in zebrafish. CB_1 receptor antagonist (rimonabant) has been reported to attenuate the salvinorin A inducing stimulation (swimming activity) of adult zebrafish (Braida et al. 2007). The zebrafish, CB1 receptor appeared in the preoptic area at 24 hour post fertilization (hpf) (Lam et al. 2006).

The cannabinoids used in this study (Δ^9-THC, WIN 55,212-2 and CP 55,940) are CB_1 and CB_2 agonists (Schatz et al. 1997, Pertwee 2008). A pronounced chronic and acute behavioral effect of cannabinoids has been observed in pubertal rats, which postulate that an immature brain could be more vulnerable to the externally exposed cannabinoid than an adult organism (Schneider et al. 2008). The aim of this study is to determine the teratology, toxicology and behavioural effects of Δ^9-THC, CP 55,940 and WIN 55,212-2 in zebrafish embryos.

Material and methods

Ethics statement

All animal experimental procedures were conducted in accordance with local and international regulations. The local regulation is the *Wet op de dierproeven* (Article 9) of Dutch Law (National) and the same law administered by the Bureau of Animal Experiment Licensing, Leiden University (Local). This local regulation serves as the implementation of *Guidelines on the protection of*

experimental animals by the Council of Europe, Directive 86/609/EEC, which allows zebrafish embryos to be used up to the moment of free-living (approximately 5-7 days after fertilization). Because embryos used here were no more than 5 days old, no license is required by Council of Europe (1986), Directive 86/609/EEC or the Leiden University ethics committee.

Animals

Male and female adult zebrafish (*Danio rerio*) of AB wild type were purchased from Selecta Aquarium Speciaalzaak (Leiden, the Netherlands) who obtain stock from Europet Bernina International BV (Gemert-Bakel, the Netherlands). The AB strain is a wild type strain (see www.zfin.org) and shows high genetic diversity, increasing the likelihood that we will detect idiosyncratic responses to the toxins. Fish were kept at a maximum density of 100 individuals in glass recirculation aquaria (L 80 cm; H 50 cm; W 46 cm) on a 14 h light: 10 h dark cycle (lights on at 08.00). Water and air were temperature controlled (25±0.5 $^{\circ}$C and 23 $^{\circ}$C, respectively). The fish were fed twice daily with 'Sprirulina' brand flake food (O.S.L. Marine Lab., Inc., Burlingame, USA) and twice a week with frozen food (artemias; Dutch Select Food, Aquadistri BV, the Netherlands).

Defined embryo buffer

To produce a defined and standardized control and vehicle for these experiments, we used 10% Hank's balanced salt solution (made from cell-culture tested, powdered Hank's salts, without sodium bicarbonate, Cat. No H6136-10X1L, Sigma-Aldrich, St Louis, MO) at a concentration 0.98 g/L in Milli-Q water (resistivity = 18.2 MΩ·cm), with the addition of sodium bicarbonate at 0.035 g/L (Cell culture tested, Sigma Cat S5761), and adjusted to pH 7.46. A similar medium has been used previously in other studies (Ali et al. 2011, Ali et al. 2011a, Ali et al. 2011b, Wielhouwer et al. 2011).

Embryo preparation

Embryo preparation was done according to Ali et al. 2011. Briefly, all incubations of embryos were carried out in an incubator with orbital shaking (50 rpm) under a light cycle of 14 h light: 10 h dark (lights on at 8.00 in the morning). The embryos were gently transferred at 24 hours post fertilization (hpf) using a sterile plastic Pasteur pipette into 96-well microtitre plates (Costar 3599, Corning Inc., NY). A single embryo was plated per well, so that embryos subsequently dying would not affect others; and also to allow individual embryos to be tracked for the whole duration of the experiment, including for behavioral recording.

Cannabinoid treatment

A significant proportion of zebrafish eggs cultured under laboratory conditions are either unfertilised or die within a few hours (Ali et al. 2011). For this reason, we began administration of cannabinoids at 24 hpf. Purification of Δ^9-tetrahydrocannabinol was done by using centrifugal partition chromatography (Hazekamp et al. 2004). The final concentration of dimethlysulphoxide (DMSO) in the water was 0.01%. All pipetting was done manually, with an 8-channel pipetter.

Preliminary range-finding

To determine a suitable range of concentrations for testing, we performed range-finding. The concentrations were in a geometric series in which each was 50% greater than the next lowest value (United States Environmental Protection Agency ,1996, Ali et al. 2011). We used 0.0, 12.5 25.0 50.0 and 100 mg/L of cannabinoids. A static replacement regime was used. Thus, there was no refreshment of buffer after the addition of compound. Each well contained 250 µL of either test compound or control (buffer only) or vehicle (0.01% DMSO in

buffer). We used 32 embryos for each concentration and 32 embryos each cannabinoid as control, and 32 embryos for each cannabinoid to control for the vehicle. The embryos for controls and treatment groups for each compound were plated in the same 96-well microtitre plates.

Mortality scoring

Mortality rate was recorded at 48, 72, 96 and 120 hpf in both range-finding and test concentration experiments, by examination under a dissecting stereomicroscope. Embryos were scored according to (Ali et al. 2011).

Refined geometric series and LC_{50} determination

After the range-finding experiments, a series of concentrations lying in the range between 0% and 100% mortality were selected for LC_{50} determination. The range for Δ^9-THC and WIN 55,212-2 was 0.3-9.6 mg/L and for CP 55,940 was 2.25-18.0 mg/L. Each geometric series of concentrations for each compound was repeated three times (in total 36 embryos per concentration and 36 embryos for vehicle for each compound). The embryos for controls and treatment groups for each compound were plated in the same 96-well microtitre plates in each independent experiment. The LC_{50} in mg/L was then converted into LC_{50} mmol/L. The LC_{50} (expressed in mg/L of buffer) was determined based on cumulative mortality at 120 hpf using Regression Probit analysis with SPSS Statistics v.17.0 (SPSS Inc., Chicago, USA).

Antagonist treatment

A geometric series of concentrations (0.5-8 mg/L) of AM251 was used to select effective concentrations for further testing. These selected concentrations were used together with one fixed concentration of each cannabinoid. The concentrations of cannabinoids selected were those on which hyper-locomotor activity was obtained.

Chronic and acute exposure

The exposure of cannabinoids for 96 h (24- 120 hpf) is defined as a chronic while for 1-12 h as acute exposure regime.

Behavioural analysis

The visual motor response test was performed at 5 days post fertilization (dpf) according to Ali et al. (2011a, 2011b) on all living larvae of both range finding experiments and geometric series. The test was performed in the presence of original solutions added at 24 h. Thus, there was no replacement or refreshment of buffer before test. The temperature used for testing was 28±0.5 °C. The visual motor response test has been previously characterized and typically consists of brief (less than 10 min) frequently alternating periods of light and dark. A key feature of this test is the robust but transient behavioral activity that occurs in response to sudden transitions from light to dark (Burgess and Granato 2007, Emran et al. 2008, Macphail et al. 2009, Rihel et al. 2010). Because such behavioral response has been shown to be highly sensitive to neuroactive chemical compounds, the visual motor response test has become a validated tool to assess the impact of a wider range of chemical agents on neuronal and physiological integrity of the developing zebrafish (Burgess and Granato 2007, Emran et al., 2008, Macphail et al. 2009, Rihel et al. 2010). Here we used a modified version of this test consisting of a single transition from light to dark. The activity of each larva was automatically recorded and analyzed in the ZebraBox recording apparatus equipped with VideoTrack software (both from Viewpoint S.A., Lyon, France). The white light intensity of the ZebraBox was 500 lux. The experimental recording consists of two steps. First, larvae were acclimated to the behavioral setup with lights ON for 2 min. This period was necessary and sufficient to ensure low and stable behavioral activity. Once basal levels of locomotor activity were stabilized following the acclimatizing period,

basal swimming activity was recorded during 4 min with lights ON. This period is referred to as 'basal context'. Immediately following the basal activity recording, the lights were suddenly turned off for 4 min. Behavioral activity in the dark was also automatically recorded during this period. This period is referred to as the 'dark challenge context'. We chose four-minute session to prevent habituation, and also to favor more robust behavioral changes. Because of the robustness of the behavioral changes induced by varying illumination, this task can be used to reveal more readily than any other tasks, defective brain function, aberrant nervous system development and/or locomotor and visual defects caused by toxic compounds (Ali et al. 2011a).

Morphological assessment of embryo phenotypes in the survivor population

Morphological assessment was done according to Ali et al. (2011a). All embryos remained in their original multi-well plates, so that every individual could be tracked throughout the entire experimental and analysis procedure. The phenotypes were scored according to the criteria listed in Table 1.

Table 1. Phenotype analysis.

Larval phenotype	Criteria
1. Normal	Absence of any of the phenotypes listed below:
2. Heart	Presence of pericardial oedema
3. Yolk	Presence of yolk sac oedema
4. Pigmentation	Dispersion of melanocytes (pigment cells)
5. Tail	Tail bent
6. Body axis	Body/primary axis bent/curved
7. Meckel's cartilage	Meckel's cartilage grossly hypoplastic, missing or unfused in midline. These effects may be unilateral or bilateral.
8. Branchial arches	One or more cartilages of the branchial skeleton hypoplastic or missing.

Description of the seven categories used to score larval phenotype at 5 dpf

Statistical analysis

Statistical analyses were performed using GraphPad Prism for Windows (version 5.03) and also used to plot graphs. To analyze the impact of compounds on embryo locomotion in the visual motor response test, we used one-way analysis of variance and a Dunnett's Multiple comparison test with probability level of 5% as the minimal criterion of significance. LC_{50} was determined using Regression Probit analysis (Chi-Squares test, Pearson Goodness-of-fit test and 95% confidence interval) with SPSS Statistics for windows version. 17.0 (SPSS Inc., Chicago, USA).

Results

LC_{50} of cannabinoids

The LC_{50} was determined for chronic exposure of zebrafish embryos to cannabinoids (96 h of exposure beginning at age 24 hpf). The following LC_{50} values were obtained at 5 dpf: Δ^9-THC, 3.37 mg/L (0.01 mmol/L); WIN 55,212-2, 1.8 mg/L (0.003 mmol/L); and CP 55,940, 16.92 mg/L (0.049 mmol/L).

Functional impairment at sub-lethal concentrations

We analysed the degree of behavioural change in zebrafish embryos exposed to cannabinoids. We used a behavioral test, the *visual motor response test*, which relies on the integrity of the central and peripheral nervous systems, including the visual system, and on normal locomotor and skeletal system development. The effects of three cannabinoids are illustrated in Figure 1and Figure 2.

We analysed the effects on total distance moved in the basal, challenge and recovery phases for both chronic and acute exposure regimes (Table 2, Table 3). The effects on this locomotory parameter fell into the categories of monotonic

stimulation; monotonic suppression; biphasic response (stimulation at lower and suppression at higher concentrations); or no significant effect.

Table 2. Concentration-dependent functional impairment by three cannabinoids (chronic exposure)

	Tdm[a] in basal phase (light on)			**Tdm in challenge phase (light off)**			**Tdm in recovery phase (light on)**		
	=	↓	↑	=	↓	↑	=	↓	↑
Compound	**Con[b] (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**
Δ^9-THC	0.3, 0.6, 1.2, 2.4	-	-	0.3, 0.6, 2.4	-	1.2	0.3, 0.6, 1.2, 2.4	-	-
WIN55,212-2	0.3, 0.6, 1.2	-	-	0.3, 0.6, 1.2	-	-	0.3, 0.6, 1.2	-	-
CP55, 940	2.25, 4.5, 9	-	-	2.25, 4.5, 9	-		2.25, 4.5, 9	-	-

Key: '=' equal to control; '↓' significantly lower than control; '↑' significantly higher than control; 'a' Total distance moved; 'b' Concentration.

Effect of chronic (96 h) exposure to cannabinoids on locomotor activity

We focus here on the dark challenge phase in order to be able to make comparisons with studies on mammals. The term 'chronic exposure' is here arbitrarily applied to 96 h of treatment since this covers the major stages of organogenesis (Kimmel et al. 1995). Compared to controls, embryos exposed chronically to all Δ^9-THC concentrations showed habituation (with increasing concentration. Only with a concentration of 1.2 mg/L was there any significant stimulation in the challenge phase with Δ^9-THC (Fig. 1A).

Table 3. Concentration-dependent functional impairment by three cannabinoids (acute exposure)

		Tdm in basal phase (light on)			**Tdm in challenge phase (light off)**			**Tdm in recovery phase (light on)**		
		=	↓	↑	=	↓	↑	=	↓	↑
Compound	**Exposure duration (hour)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**
Δ^9-THC	1	0.6, 1.2	-	2.4, 3.4	0.6, 3.4	-	1.2, 2.4	0.6	-	1.2, 2.4 3.4
	4	0.6, 1.2, 3.4	-	2.4	0.6, 1.2, 2.4	3.4	-	0.6, 1.2, 3.4	-	2.4
	12	0.6, 1.2, 2.4	3.4	-	-	2.4, 3.4	0.6, 1.2	0.6, 1.2, 2.4	3.4	-
WIN 55,212-2	1	-	0.6, 1.2, 1.8	-	0.6	-	1.2, 1.8	0.6, 1.2, 1.8	-	-
	4	0.6, 1.8	-	1.2	0.6, 1.8	-	1.2	0.6, 1.2, 1.8	-	-
	12	0.6, 1.2, 1.8	-	-	0.6, 1.8	-	1.2	0.6, 1.2, 1.8	-	-
CP55, 940	1	2.25, 4.5, 9	-	18	-	-	2.25, 4.5, 9, 18	2.25, 4.5, 9	-	18
	4	2.25, 4.5, 9, 18	-	-	-	-	2.25, 4.5, 9, 18	2.25, 9, 18	-	4.5
	12	18	-	2.25, 4.5, 9	-	-	2.25, 4.5, 9,	2.25, 9, 18	-	4.5

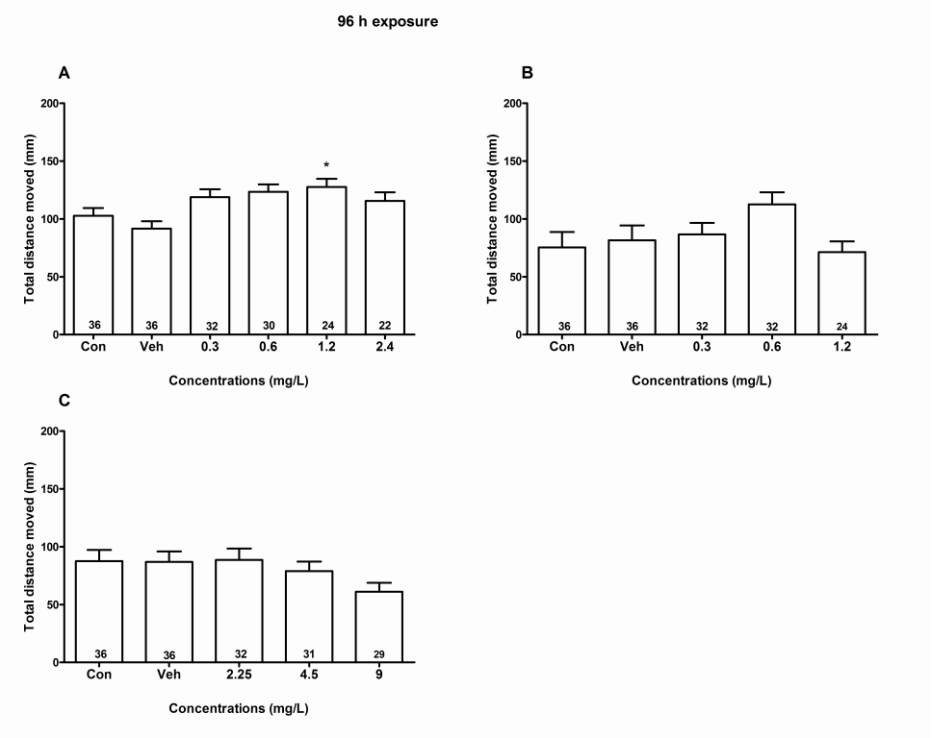

Figure 1. Behavior analysis of live zebrafish embryos treated with Δ^9-THC, WIN 55,212-2 and CP 55,940 for 96 h. **A**, locomotor activity induced by Δ^9-THC exposure; **B**, by WIN 55,212-2; **C**, by CP 55,940. ***** depict differences between controls and different used concentrations. In figures **A-C**, it can be seen that there is habituation that occurs with 96 h exposure. The number inside the base of the bars = *N* embryos .Statistical icons: *= $p < 0.05$.

Effect of acute exposure to cannabinoids on locomotor activity

Here, acute exposure is arbitrarily applied to a 1-12 h exposure starting at 108 hpf. For behavioral analysis, embryos were exposed at 4.5 dpf for 12 h, and at 5 dpf for 1-4 h (in order to provide a common endpoint of 5 d). With Δ^9-THC, there was an effect of both concentration and duration of exposure on locomotor activity. With ≥1 h exposure time, locomotor activity was stimulated at low concentrations (Fig. 2A); no effect was found with high concentrations, even after 4 h of treatment (Fig. 2B). A biphasic response (stimulation at low

concentrations and suppression at high concentrations) was found with 12 h exposure (Fig. 2C). By contrast, low concentrations continued to cause hyper-activity at 12 h exposure (Fig. 2A-C). Concerning the other cannabinoids, the action of WIN 55,212-2 on locomotor activity closely resembles that of Δ^9-THC and a biphasic response was found after 12 h of exposure (Fig. 2D-F). At low concentrations, CP 55,940 (Fig. 2G-I) and WIN 55,212-2 both gave a similar behaviour pattern as Δ^9-THC (hyper-activity from 1 h - 12 h of exposure).

Effect of exposure to the cannabinoid receptor antagonist AM251 on locomotor activity

We exposed zebrafish embryos to AM251 for 1-12 h (acute exposure) or 96 h chronic exposure. Concentration-dependent suppression of locomotor activity was found in both cases (Fig. 3A-D).

Embryos were co-exposed acutely to a cannabinoid plus antagonist (AM251) for 1-12 h. The antagonist caused a dose-dependent amelioration of the locomotor activity induced by the cannabinoid alone (Fig. 4A-I).

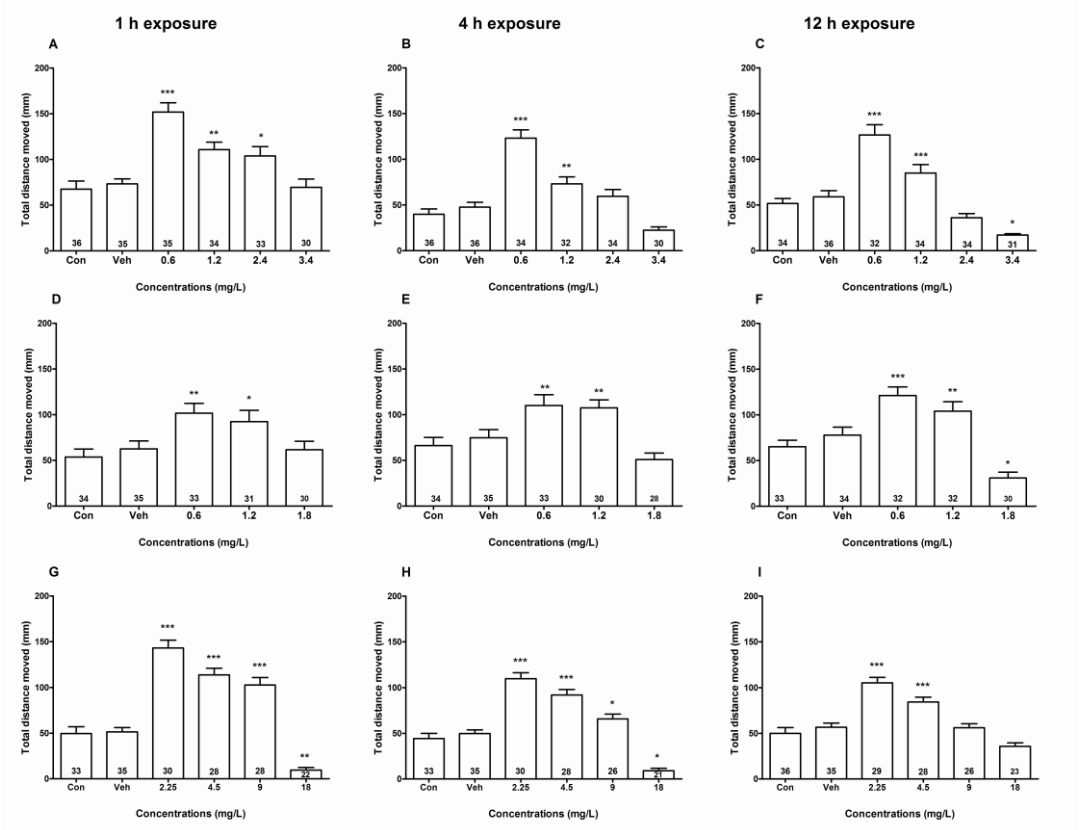

Figure 2. Behavior analysis of live zebrafish embryos treated with Δ^9-THC, WIN 55,212-2 and CP 55,940 for selected time points**.** The graphs represent locomotor activity at the following time points: **A**, **D** and **G** 1 h exposure with Δ^9-THC, WIN 55,212-2 and CP 55,940 respectively; **B**, **E** and **H** 4 h exposure with Δ^9-THC, WIN 55,212-2 and CP 55,940 respectively; **C**, **F** and **I** 12 h exposure with Δ^9-THC, WIN 55,212-2 and CP 55,940 respectively. ***** depict differences between controls and different used concentrations. In figures C, F and G, it can be seen that there is a biphasic response in acute regimes. The number inside the base of the bars = *N*. Statistical icons: *= $p< 0.05$, **= $p< 0.01$ and ***= $p< 0.001$.

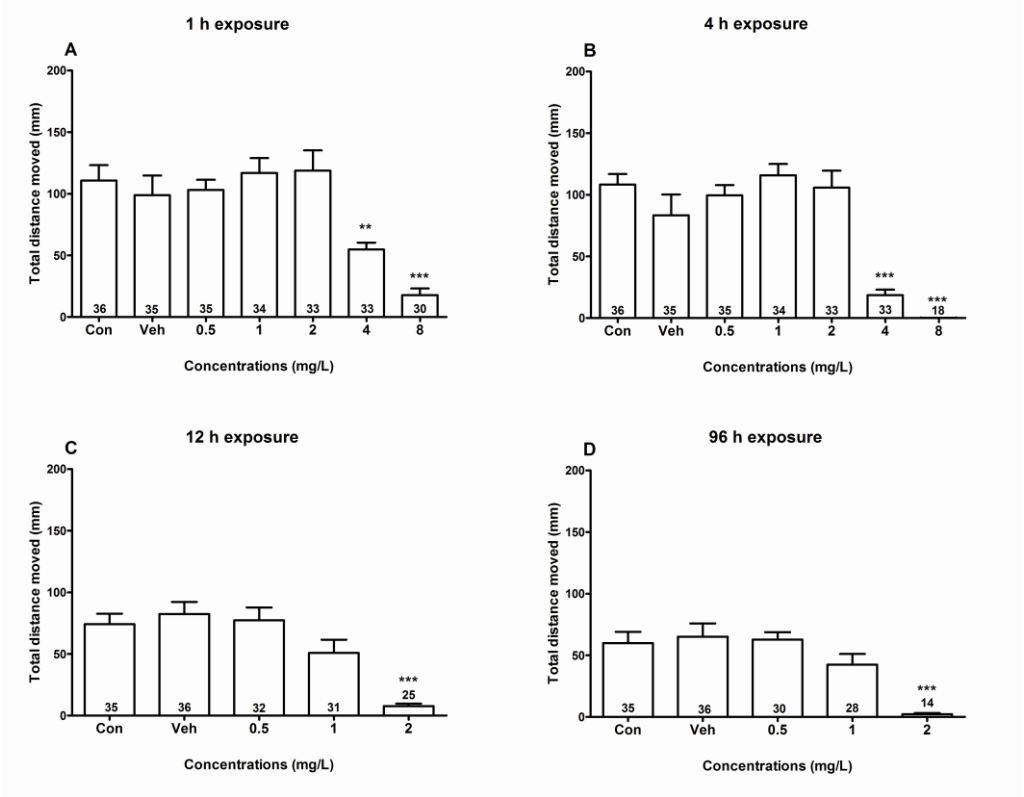

Figure 3. Behavior analysis of live zebrafish embryos treated with AM251 for selected timepoints. The graphs represent locomotor activity at the following time points: **A**, 1 h exposure **B**, 4 h; **C**, 12 h; **D**, 96 h. ***** depict differences between controls and different used concentrations. The number inside the base of the bars = *N*. Statistical icons: *= p< 0.05, **= p< 0.01 and ***= p< 0.001.

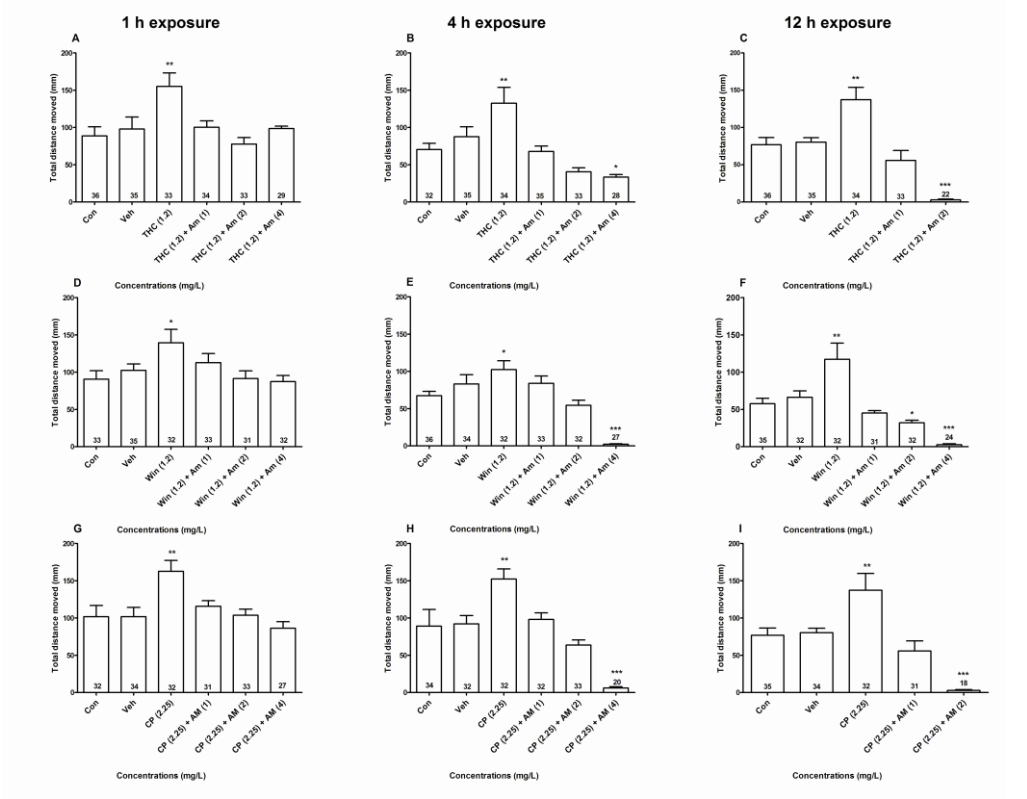

Figure 4. Behavior analysis of live zebrafish embryos co-administrated of AM251 with Δ^9-THC, WIN 55,212-2 and CP 55,940 for selected timepoints. The graphs represent locomotor activity at the following time points: A, D and G 1 h exposure to AM251 with Δ^9-THC, with WIN 55,212-2 and with CP 55,940 respectively; B, E and H 4 h exposure to AM251 with Δ^9-THC, with WIN 55,212-2 and with CP 55,940 respectively; C, F and I 12 h exposure to AM251 with Δ^9-HC, with WIN 55,212-2 and with CP 55,940 respectively. ***** depict differences between controls and different used concentrations. The number inside the base of the bars = *N*. Statistical icons: *= p< 0.05, **= p< 0.01 and ***= p< 0.001.

Morphological assessment of embryos

The results of morphological analysis of embryos are shown in Figure 5 and Table 4. With 0.3 to 2.4 mg/L Δ^9-THC treatment, the frequency of pericardial and yolk sac oedemas, and bent body, were significantly higher than in control (buffer only) and vehicle (DMSO and buffer only) experiments. With CP 55,940 and WIN 55,212-2 no significant increase in the frequency of any malformation was seen (the apparent increase in yolk sac oedema with CP 55,940 exposure was not statistically significant in view of the number of cases).

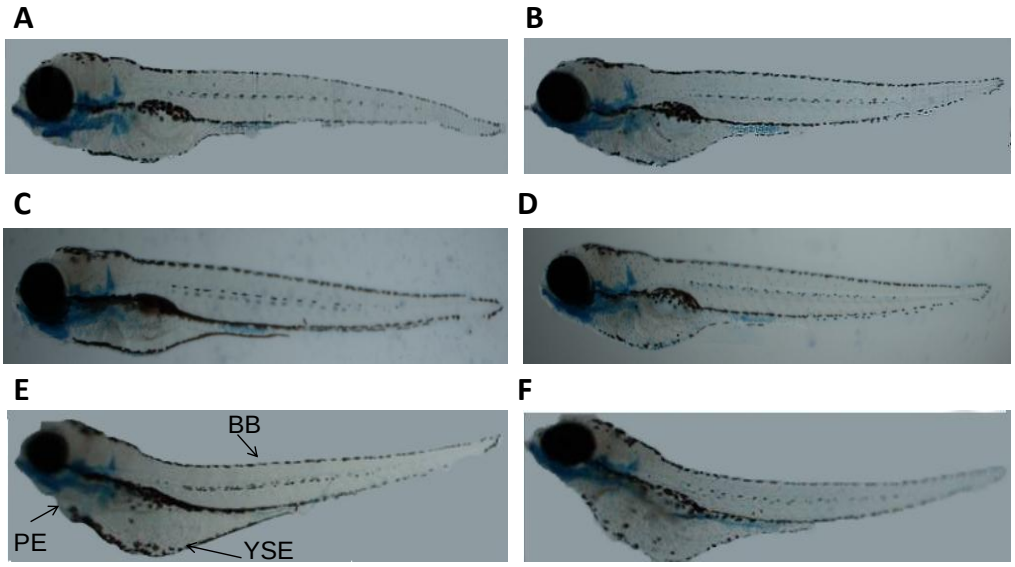

Figure 5. Morphological assessment of zebrafish embryos at 5 dpf treated with cannabinoids. Zebrafish embryos stained with alcian blue reveals the ventral view. The aim of this figure is to show the effects on body axis of embryos after 96 h exposure to cannabinoids. A, control; B, vehicle; C, 1.2 mg/L Win 55,212-2; D, 9 mg/L CP 55,940; E, 0.6 mg/L Δ^9-THC; F, 2.4 mg/L Δ^9-THC. PE, pericardial edema; YSE, yolk sac edema; BB, bent body.

Table 4. Statistical analysis of incidence of malformations in zebrafish embryos at different concentrations of Δ^9-THC.

	Significance level			
Categories	0.3 (mg/L)	0.6 (mg/L)	1.2 (mg/L)	2.4 (mg/L)
Pericardial Oedema	-	**	*	*
Yolk sac oedema	***	***	***	***
Bent tail	-	-	-	-
Body/primary axis (bent/curved)	***	***	*	*
Meckel's cartilage(hypoplasia)	-	-	-	-
Branchial arches (hypoplasia)	-	-	-	-

Key:* Statistical icons: (-), not significant; (*), $p< 0.05$; (**), $p< 0.01$; and (***), $p< 0.001$. Note that there were no significant increases in malformations after exposure to WIN 55,212 and CP 55,940. Statistical icons: *= $p< 0.05$, **= $p< 0.01$ and ***= $p< 0.001$.

Discussion

We have studied the effects of cannabinoids on the survival, locomotor activity and morphological development of zebrafish embryos. Our readouts were mortality recording, the visual motor response test and morphological analysis. Both acute and chronic exposure regimes, and the effects of the cannabinoid receptor antagonist (AM251), were examined.

In acute regimes, Δ^9-THC showed a biphasic response with increasing hyperactivity succeeded by suppression of activity as the dose increased. These findings are consistent with studies in rodents which reported a stimulation of locomotor activity by Δ^9-THC at low concentrations, and suppression at higher

concentrations (Grisham and Ferraro 1972, Ferraro and Gluck 1974, Taylor and Fennessy 1977). A recent study (Freedland et al. 2002) suggested that high concentrations of Δ^9-THC in adult rats decreased cerebral metabolism. According to this study, this metabolic change was associated with the biphasic motor behaviour of Δ^9-THC. In zebrafish embryos, CB1 receptors are expressed in the preoptic area by 24 hpf (Lam et al. 2006). We therefore chose this time to begin chronic administration of cannabinoids. For acute exposure, we began to expose embryos of 4.5 dpf so that all embryos, regardless of treatment, were analysed at the same endpoint (day 5).

WIN 55,212-2 also caused a biphasic response in acute regimes. This is consistent with findings (Drews et al. 2005) in the open field test, where rats treated with low concentrations of WIN 55,212-2 covered more distance than controls, and those treated with high concentrations covered less distance. CP 55,940 also caused a biphasic response in acute exposure regimes. Biphasic locomotor activity has been reported in rats exposed to CP 55,940 (McGregor et al. 1996). Furthermore, a pre-treatment of CP 55,940 caused hyperactivity in rats subsequently exposed to morphine (Norwood et al. 2003).

In chronic regimes, all three cannabinoids showed habituation. It is interesting to notice that the habituation is probably not accompanied by general sedation of the embryos because their locomotor activity in the corresponding basal phase is normal (Table 2, Table 3). Several studies in different species have shown that chronic exposure of cannabinoids is accompanied by the development of tolerance to many of the acute effects. These effects include memory disorder, hypothermia and analgesia (reviewed by (Howlett et al. 2004). In rodents, the development of tolerance to motor-behavioural effects of chronic cannabinoids exposure has been studied. For example, chronic exposure to Δ9-THC (Abood et al. 1993, Rodriguez et al. 1994, Howlett et al. 2004),

WIN 55,212-2 (Martini et al. 2010) and CP 55,940 (Costa et al. 1996, Rubino et al. 1997) all caused tolerance to the effects of those cannabinoids on suppressing locomotor activity. This phenomenon was associated with down regulation of CB receptors after long-term exposure to cannabinoids (Abood et al. 1993, Rodriguez et al. 1994, Costa et al. 1996, Rubino et al. 1997, Howlett et al. 2004, Martini et al. 2010). Hence, the study of CB receptors expression level after a chronic exposure to cannabinoids can further extend our understanding of the phenomenon of tolerance in zebrafish embryos.

An increased incidence of curved body axis and bent tail were found in embryos exposed chronically to Δ^9-THC. It is necessary, therefore to consider the possibility that the changes in locomotion and behaviour were caused by these malformations, and not by an action of the cannabinoid on the nervous system. But it can be seen in Figure 1, embryos exposed chronically to Δ^9-THC have shown similar locomotor activity compared to control. Moreover, Win 55,212-2 and CP 55,940 have not shown any significant incidence of malformations at any concentration tested, yet do show changes in locomotor behaviour in acute regimes. This suggests that the locomotor effects of these cannabinoids is not a secondary one due to teratogenicitiy.

It has previously been reported in rodents that AM251 decreases the total distance travelled in open field test (Sink et al. 2010) and also blocks the locomotor excitation caused by CB_1 agonists (Kongkam et al. 2008). Our data suggest that AM251 attenuates the increased locomotor activity induced by CB_1 agonists. These results implicate the involvement of CB_1 receptors in the regulation of locomotor activity in zebrafish larvae and are in good agreement with previous rodent studies.

It has also been shown that AM251 attenuates the behavioural sensitization induced in rodents by amphetamine, nicotine and Δ^9-THC (Gatley et al. 1996,

Thiemann et al. 2008, Le et et al. 2008). These studies show that the blockade of CB1 receptor not only opposes the inducing effect of cannabinoids but can also alter the activity of other psychotropic compounds having binding sites other than CB receptors. Another study found that CP 55,940 has one, and WIN 55212-2 two different binding sites in the zebrafish brain (Rodriguez et al. 2007). So, It would be of great interest to explore the AM251 binding affinity in the zebrafish brain. It is also likely that CB receptor-knockout in zebrafish embryos will lead to a deeper understanding of the role of CB receptors in zebrafish physiology. Moreover, study of agonist and antagonist interactions could be helpful in understanding the zebrafish endogenous cannabinoid system.

Conclusions

Our findings show that 96 h duration of exposure in zebrafish embryos starting at 24 hpf can be used to study the teratology of sub-lethal concentrations of cannabinoids. This regime also leads to habituation in behavioural response. In acute exposure, our findings are similar to the results found in rodents, with dose-dependent hyperactivity followed by suppression. The antagonist blocks the increased locomotor activity induced by cannabinoids. This suggests that some similarity in cannabinoid response pathways between zebrafish and mammals exists. Further validation, and study of receptor interactions, is needed before we can be sure that the zebrafish embryo can be a useful tool for the pre-clinical screening of natural, synthetic and endogenous cannabinoids.

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