

Metabolic effects of cannabinoids in zebrafish (*Danio rerio*) embryos determined by ^1H NMR metabolomics

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Abstract The study of drug-driven biochemical changes is important in order to determine the biomarkers associated with a specific compound activity in an individual biological system. Rodent models have been widely used to study the metabolic changes induced by psychostimulants in a cell, tissue or whole organism. However these models are not suitable for large-scale, high-throughput screening. Here, we used zebrafish embryos to study the metabolic effects of the cannabinoid receptor type 1 (CB₁) agonist (–)-(6aR,10aR)-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6H-benzo[c]-chromen-1-ol (Δ^9 -THC) and antagonist (AM251). The zebrafish embryos were exposed to Δ^9 -THC and AM251 at 24 h post fertilization (hpf) for 96 h. Proton nuclear magnetic resonance based metabolomic results show an increase in the level of choline, betaine, taurine, adenosine triphosphate and glucose upon exposure to Δ^9 -THC. The levels of excitatory neurotransmitters (glutamate and glutamine) increased at lower doses of Δ^9 -THC, whereas toxic dose resulted in reduction of glutamate. In contrast to Δ^9 -THC, AM251

caused a dose dependent reduction of betaine, choline, taurine and also reduce the level of glutamate and glutamine. Interestingly, both compounds induce the production of the dopamine precursors, phenylalanine and tyrosine at higher doses. These findings suggest that CB₁ receptor is involved in the regulation of metabolites, which might be involved in the neurotransmission of zebrafish embryos. Furthermore, our results show that zebrafish embryo can be successfully used to provide a detailed overview of general effects of drug on the overall metabolome of an intact organism.

Keywords Zebrafish embryo · Metabolomics · Predictive model · Cannabinoids · Cannabinoid receptor · Antagonist

1 Introduction

Δ^9 -tetrahydrocannabinol (Δ^9 -THC) is a psychoactive compound (Pamplona and Takahashi 2012) and one of the major cannabinoids present in *Cannabis Sativa* (Yamaori et al. 2011). Many pharmacological activities of Δ^9 -THC are attributed to its psychoactive properties (Costa 2007). In turn, many of these activities depend on the interaction of Δ^9 -THC with cannabinoid receptors (Lichtman and Martin 1996). Since the discovery of cannabinoid receptors (Matsuda et al. 1990; Munro et al. 1993), a great deal of work has been done to elucidate their role in cannabinoid-mediated activities (Alhamoruni et al. 2012; Fraga et al. 2011; Karmaus et al. 2011).

The zebrafish embryo is emerging as a useful animal model for high throughput screening of new drugs. Small size, ease of maintenance, short reproductive cycle and optical transparency are the main features, which make zebrafish embryos an attractive tool for the preclinical screening of bioactive compounds (Ali et al. 2011; Ingham

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2009; Mandrekar and Thakur 2009). The presence of CB receptors (Migliarini and Carnevali 2009; Rodriguez-Martin et al. 2007) in zebrafish embryos provide a rationale to study natural, synthetic and endogenous cannabinoid regulated activities in zebrafish.

Rodent models have been used to study the behavioral and pharmacological effects of cannabinoids (Rubio et al. 1995; Wiley et al. 2007; Wise et al. 2011). Biphasic behavioral and physiological responses have been reported in rodents subjected to Δ^9 -THC (Grisham and Ferraro 1972; Stiglick and Kalant 1982; Taylor and Fennessy 1977). This biphasic response is connected with local cerebral glucose utilization in rats, where administration of a higher dose of Δ^9 -THC slowed down cerebral metabolism in most brain regions, whereas no significant decrease was found in animals exposed to lower doses of Δ^9 -THC (Freedland et al. 2002). These biphasic responses have also been reported in the level of brain monoamines and body temperature (Taylor and Fennessy 1977). Recently, Akhtar et al. (2013) have studied the effects of Δ^9 -THC and two CB₁ receptor agonists (WIN 55,212-2 and CP 55,940) on the behavior and morphology of zebrafish embryos. Interestingly, they found a biphasic behavioral response in an acute exposure [1–12 h exposure starting at 108 h post fertilisation (hpf)] in zebrafish embryos similar to that reported in rodents (Grisham and Ferraro 1972). In view of these findings, we decided to investigate the cannabinoid induced metabolic effects in 5dpf zebrafish embryos.

Metabolomics is a comprehensive study of a collection of the metabolites present in a cell, tissue, or organism at a particular time (Deidda et al. 2015). It is a very useful tool in research efforts aimed at understanding the metabolism and related physiological processes at a systems biology level (Weckwerth 2003). High-resolution proton nuclear magnetic resonance (¹H NMR) is an ideal tool for the metabolite profiling of biofluids, tissue extracts and intact tissues (Griffin 2004). ¹H NMR spectroscopy has been used to investigate the biochemical composition of different tissues in an organism (Coen et al. 2003; Griffin 2003; Jones et al. 2007) and drug toxicity assessment in the serum, liver and testis of rodents (Atherton et al. 2006; Ekman et al. 2006; Griffin et al. 2007; Waters et al. 2006).

Previously, a high performance liquid chromatography (HPLC) method was used to analyze the levels of excitatory and inhibitory amino acids in the brain tissue of rats treated with Δ^9 -THC (Hikal et al. 1988). Here, in order to investigate a wide range of metabolites, we used NMR based metabolomics to study the effects of CB receptor type 1 (CB₁) agonist Δ^9 -THC and CB₁-antagonist AM251 on metabolic profile of 5 days post fertilization (dpf) zebrafish embryos. The aim of the present study is to validate zebrafish embryo as a model to investigate cannabinoid-associated metabolomic effects in a whole organism.

2 Materials and methods

2.1 Ethics statement

All animal experimental procedures were conducted in accordance with national regulations, which are described in the *Wet op de dierproeven* (article 9) of Dutch Law administered by the Bureau of Animal Experiment Licensing. This national regulation serves as the implementation of “Guidelines on the protection of experimental animals” by the Council of Europe (1986), Directive 86/609/EEC, which allows zebrafish embryos to be used up to the moment of free-living (approximately 5–7 dpf). 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.

2.2 Zebrafish

Male and female adult zebrafish (*Danio rerio*) of wild type were purchased from Selecta Aquarium Specialzaak (Leiden, The Netherlands) which obtain stock from Europet Bernina International BV (Gemert-Bakel, The Netherlands). The 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 and 23 °C, respectively). All the zebrafish handling was in accordance with national regulations. The fish were fed twice daily with ‘Sprirulina’ brand flake food (O.S.L. Marine Lab Inc and Burlingame, CA, USA) and twice a week with frozen food (Dutch Select Food, Aquadistri BV, The Netherlands).

2.3 Defined embryo buffer

Ten percent (v/v) of Hank’s balanced salt solution (HBSS) was used (made from cell-culture tested, powdered Hank’s salts, without sodium bicarbonate, Cat. No H6136-10X1L, Sigma-Aldrich, St Louis, MO, USA) 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), and adjusted to pH 7.46. A similar medium has been used previously used (Irons et al. 2010; Macphail et al. 2009).

2.3.1 Embryo care

Eggs were obtained by random pair wise mating of zebrafish. Three adult males and four females were placed together in small breeding tanks (Ehret GmbH, Emmendingen, Germany) the evening before eggs were required. The breeding tanks (L: 26 cm, H: 12.5 cm, W: 20 cm) had mesh

egg traps to prevent the eggs from being eaten. The eggs were harvested the following morning and transferred into 92 mm plastic Petri dishes (50 eggs per dish) containing 40 ml fresh embryo buffer. Eggs were washed four times to remove debris. Further unfertilized, unhealthy and dead embryos were screened under a dissecting microscope. At 3.5 h post fertilization (hpf), embryos were again screened and any further dead and unhealthy embryos were removed. Throughout all procedures, the embryos and the solutions were kept at 28.5 °C, in acclimatised room. All incubations of embryos were carried out in acclimatised room under a light cycle of 14 h light: 10 h dark.

2.4 Preliminary range-finding

To determine a suitable range of concentrations of testing compounds, we performed range-finding. The concentrations were in a geometric series in which each was 50 % greater than the next lowest value. We used 0.0, 12.5, 25.0, 50.0 and 100 mg/L of Δ^9 -THC and AM251. A static replacement regime was used. Thus, there was no refreshment of buffer after the addition of compound. Each well contained 250 μ L of either test compound or control (HBSS) or 0.01 % Dimethyl sulfoxide (DMSO) dissolved in HBSS as a vehicle. We used 32 embryos for each concentration, 32 embryos for 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.

2.4.1 Geometric series and LC_{50} determination

After the range-finding experiments, a series of sublethal concentrations were selected for LC_{50} determination. The range for Δ^9 -THC and AM251 was 0.3–9.6 and 0.5–8 mg/L, respectively. 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} (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, IL, USA).

2.5 Embryo treatment and collection

One hundred and twenty embryos per replicate were collected. Sixty embryos per 100 \times 15 mm Petri dish were cultured. After 24 h the embryos were treated with 0.6, 1.2, 2.4 and 3.4 mg/L (LC_{50}) of Δ^9 -THC; 0.6, 1.2, 2.4 and 4.8 mg/L (LC_{50}) of AM251. At 5dpf, embryos were collected in a

Falcon tube. The collected embryos were immediately frozen in liquid nitrogen and stored at -80 °C until further analysis.

2.6 Sample preparation and extraction

A fairly simple sample preparation method was followed which has already been used in plant metabolomics (Abdel-Farid et al. 2009; Ali et al. 2010; Jahangir et al. 2008; Kim et al. 2010a, b). Embryos were freeze-dried and transferred to an Eppendorf tube (2 ml) to which 1 ml of 50 % CH_3OH - d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.01 % trimethylsilylpropionic acid (TMSP) (w/w) was added. The mixture was vortexed for one min, sonicated for 20 min, and centrifuged at 13,000 rpm at room temperature for 10 min. An aliquot (650 μ L) was then transferred to a 5 mm-NMR tube. The NMR spectrum was recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. CH_3OH - d_4 was used as the internal lock. Each 1H NMR spectrum consisted of 128 scans according to parameters used in our group (Kim et al. 2010b).

2.7 Data pre-processing and analysis

The intensities of 1H -NMR spectra were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.4– δ 10.0. The regions of δ 4.8– δ 4.9 and δ 3.30– δ 3.34 were excluded from the analysis because of the residual signal of the deuterated solvents. Principal component analysis (PCA) was performed with SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) based on a Pareto scaling method. All of the spectra were referenced, base line corrected, phase-corrected and visually inspected by superimposing and stacking the spectra (Fig. 1a) using MestReNova v.6.0.2 (Mestrelab research S.L., A Coruna, Spain).

3 Results and discussion

3.1 Principal component analysis

We applied principal component analyses (PCA) to evaluate the variables representing the key metabolites regulated differently in the Δ^9 -THC treated and non-treated zebrafish embryos. The internal cross validation of the PCA revealed that four components were enough to represent 84 % variation in the data. Inspection of score plot shows that 40.6 % of the separation is based on PC1 and 21.8 % is based on PC2 scores (Fig. 1b). The major source

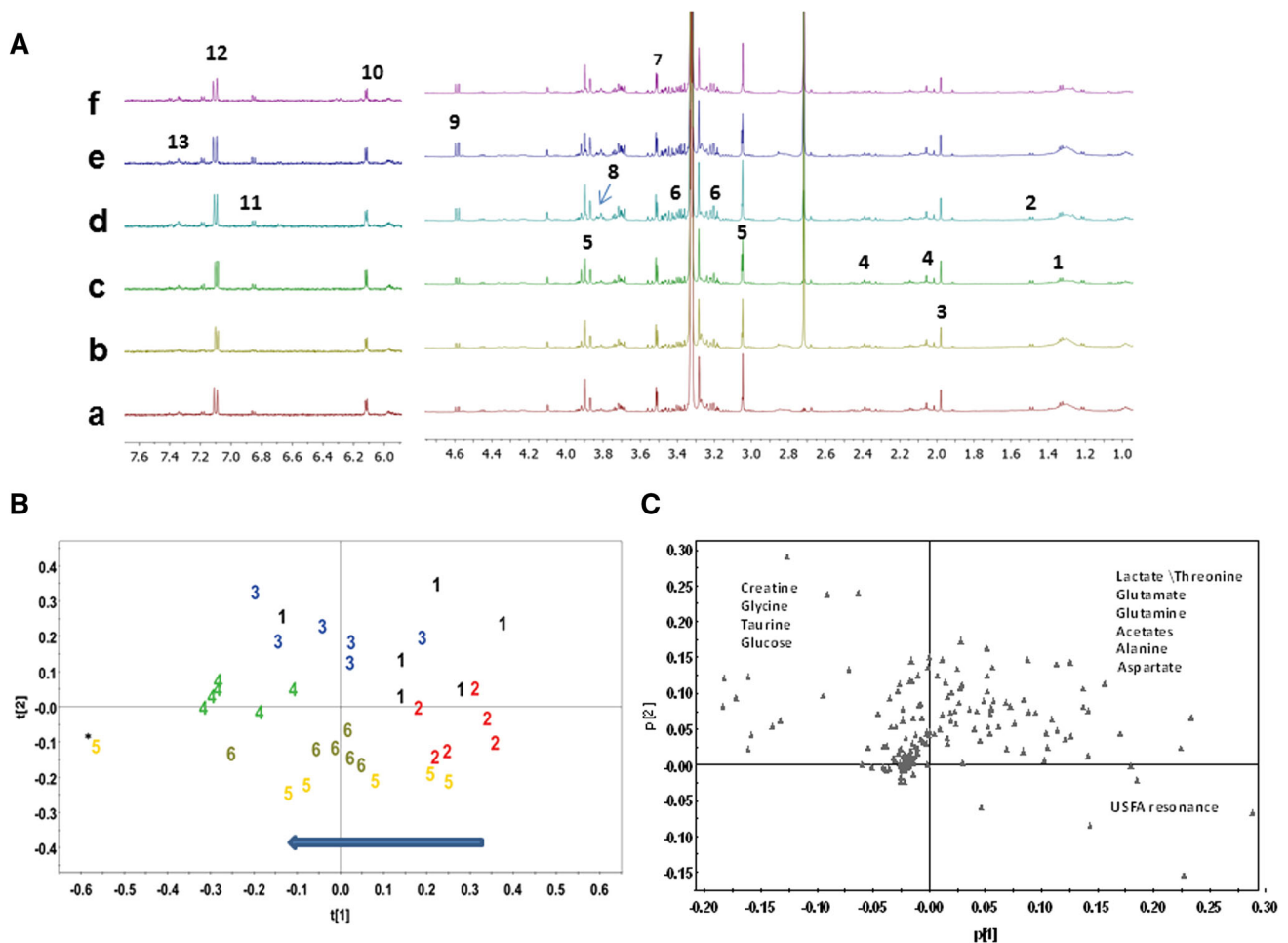


Fig. 1 **a** Comparison of ^1H NMR spectra of zebrafish embryos, showing the region of interest after treating the embryos with Δ^9 -THC. **a** control, **b** 0.01 % DMSO, **c** 0.6 mg/L Δ^9 -THC, **d** 1.2 mg/L Δ^9 -THC, **e** 2.4 mg/L Δ^9 -THC, **f** 3.4 mg/L Δ^9 -THC. **1** lactate **2** alanine, **3** acetate, **4** glutamate **5** choline, **6** taurine, **7** glycine, **8** betaine, **9** creatine, **10** glucose, **11** ATP, **12** tyrosine, **13** histidine, **14**

phenylalanine. **b** Scatter plot from principal component analysis; the figures shows a trend from low to high doses as indicated by the blue arrow. **1** Control, **2** 0.01 % DMSO, **3** 0.6 mg/L Δ^9 -THC, **4** 1.2 mg/L Δ^9 -THC, **5** 2.4 mg/L Δ^9 -THC, **6** 3.4 Δ^9 -THC; asterisk represent the outlier. **c** Representative loading plot of PCA analysis

of variability among the samples in score plot is strongly correlated with the dose of Δ^9 -THC (Fig. 1c). The control (HBSS) and vehicle (0.01 % DMSO) groups tend to cluster together along the positive side of PC1 (horizontal line), while the most affected group clustered far from the control is the medium dose group (1.2 mg/L). The high dose (2.4 and 3.4 mg/l) groups cluster together in between low and medium doses (Fig. 1b). The corresponding loading plot shows that the levels of lactate, glutamate, glutamine, acetate, alanine and aspartate are associated with the embryos treated with Control, DMSO and lowest dose (0.6 mg/L) of Δ^9 -THC. Whereas, creatine, taurine and glucose contents were increased in the groups treated with medium (1.2 mg/L) and higher doses (2.4 and 3.4 mg/L) of Δ^9 -THC (Fig. 1c). The relative quantification of important metabolites is shown in supplementary Fig. 1a.

3.2 Orthogonal partial least square (PLS) analysis

The NMR data were subjected to orthogonal partial least square analysis. A 'Y' variable was included in the data which represents the different classes (control, 0.01 % DMSO treated, low dose (0.6 mg/L Δ^9 -THC), medium dose (1.2 mg/L Δ^9 -THC), high dose (2.4 mg/L Δ^9 -THC) and LC₅₀ (3.4 mg/L Δ^9 -THC).

PLS modeling was applied because less variation and discrepancy appeared in the score plot (Fig. 1b) which might be because of un-correlated variables (metabolites), noise or systematic variations in NMR data effecting the separation in score plot. To remove these un-correlated data we developed a model with the use of a data filter called orthogonal signal correction (OSC). This filter has already been applied successfully in numerous studies

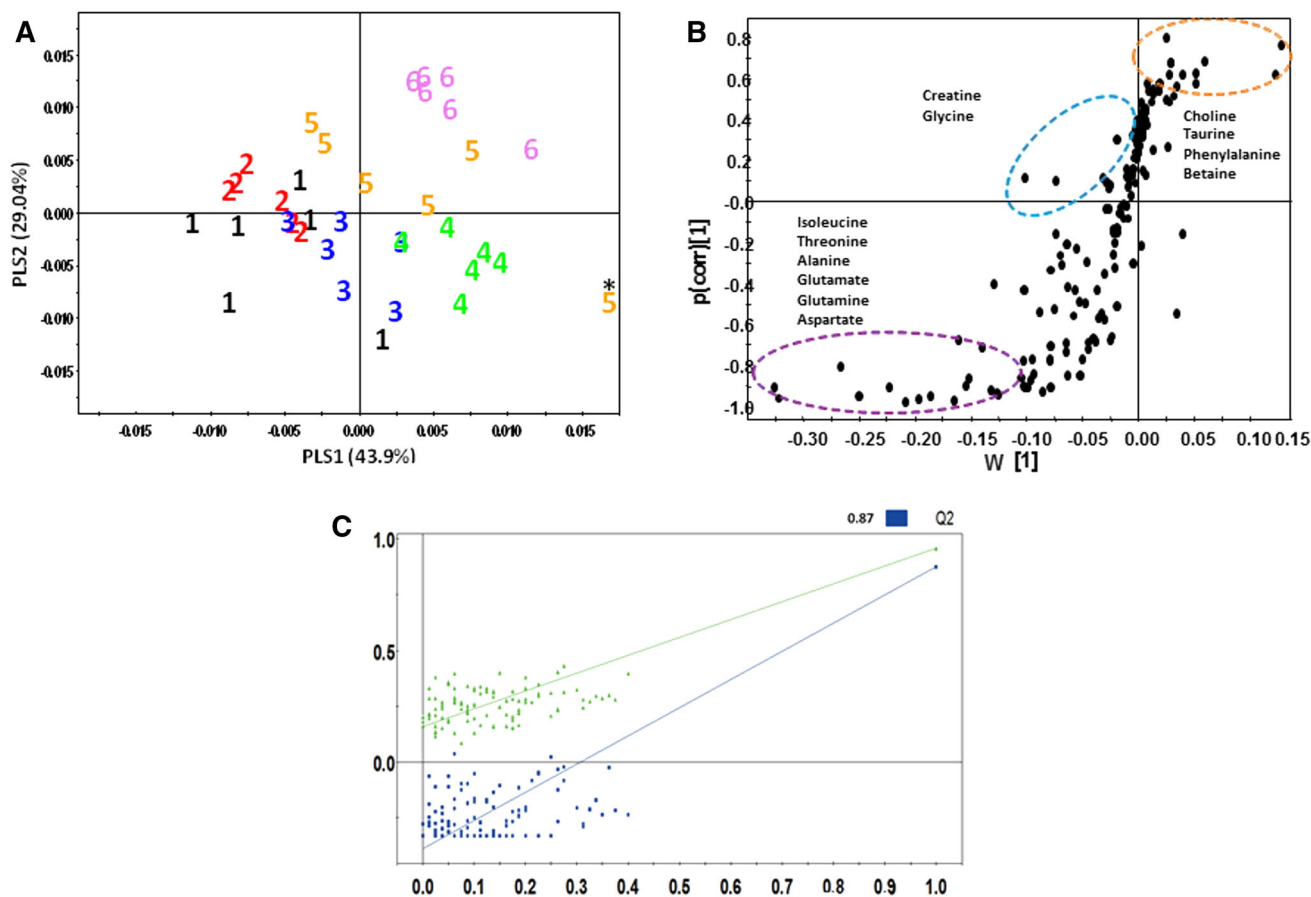


Fig. 2 **a** Scatter plot of partial least square analysis with an orthogonal signal correction filter. 1 Control, 2 0.01 % DMSO, 3 0.6 mg/L Δ^9 -THC, 4 1.2 mg/L Δ^9 -THC, 5 2.4 mg/L Δ^9 -THC, 6 3.6 mg/L Δ^9 -THC. Asterisk the outlier. **b** S-Plot shows the markers

(variables) responsible for the separation. The *upper right corner* corresponds to the higher doses of Δ^9 -THC while the *lower left corner* corresponds to the lower doses and control group of Δ^9 -THC. **c** Validation of OPLS with permutation test

(Gavaghan et al. 2002; Hauksson et al. 2001). The improvement in the model can be seen in Fig. 2c where comparison of the cross validation of two components is shown. After applying the filter, three components of the model were able to describe 95 % of the variation. The model was validated using a permutation test with 100 permutations as shown in Fig. 2c. The results indicate a good validation of the model as the intercept value of both R2Y and Q2Y are within the limits as described by Eriksson et al. 2006. The scatter plot from this data shows the grouping of control, DMSO and low dose (0.6 mg/L) treated groups on the negative side of PLS1, while the moderate dose (1.2 mg/L) clustered on positive side of PLS1 and negative side of PLS2 (Fig. 2a). The highest dose (3.4 mg/L) group, on the other hand, belongs to the positive side of both PLS1 and PLS2.

The corresponding loading plot or S-plot shows the variables affecting the separation in the scatter plot (Fig. 2b). The variables (^1H NMR-chemical shifts) related to certain key metabolites were identified and are shown in

Table 1. The identification of metabolites confirms that the levels of amino acids including glutamate, glutamine, isoleucine, alanine, threonine, aspartate, taurine, phenylalanine, choline, creatine and glycine are affected in embryos treated with Δ^9 -THC. The comparison of the S-plot with the scatter plot reveals that the lower levels of alanine, threonine, aspartate, glutamate and glutamine are associated with the embryos treated with the highest dose (3.4 mg/L) of Δ^9 -THC. Whereas the quantity of phenylalanine, taurine and choline was increased in the samples treated with higher doses of Δ^9 -THC compared to the control group. The visual analysis of ^1H NMR spectra reveals that different doses of Δ^9 -THC resulted in differential changes in the levels of metabolites in the embryos (Fig. 1a). The levels of glucose, acetate and Adenosine triphosphate (ATP) elevated with the increasing concentration (0.6–2.4 mg/L) of Δ^9 -THC and subsequently decreases at the highest concentration of Δ^9 -THC (3.4 mg/L). A dose-dependent increase was observed in the level of choline, glycine and betaine. Phenylalanine and tyrosine

Table 1 Characteristic ^1H NMR chemical shifts and coupling constants of identified metabolites in 5 days old zebrafish larvae

No	Metabolites	Chemical shifts (δ) and coupling constant	Accession no.
1	Acetate	δ 1.91 (s)	HMD00042
2	Alanine	δ 1.48 (d, $J = 7.1$ Hz)	HMDB00161
3	ATP	δ 6.13 (d, $J = 5.0$ Hz), δ 8.26 (s), δ 8.56 (s)	HMDB00538
4	Betaine	δ 3.29 (s)	HMDB00043
5	Creatine	δ 3.06 (s), δ 3.91 (s)	HMDB00064
6	Choline	δ 3.23 (s)	HMDB00097
7	Glucose	δ 4.60 (d, $J = 7.9$ Hz), δ 5.20 (d, 4.0 Hz)	HMDB00122
8	Glutamate	δ 2.05 (m), δ 2.40 (m), δ 3.71 (m)	BMSE001013
9	Glutamine	δ 2.14 (m), δ 2.46 (m), δ 3.71 (m)	HMDB00641
10	Glycine	δ 3.52 (s)	HMDB00123
11	Histidine	δ 7.07 (d, $J = 0.6$), δ 7.84 (d, $J = 1.1$)	HMDB00177
12	Lactate	δ 1.32 (d, $J = 7.0$), δ 4.06 (m)	HMDB00190
13	Leucine	δ 0.99 (d, $J = 7.0$), δ 1.00 (d, $J = 7.0$), δ 1.68 (m), δ 3.68 (m)	BMSE000042
14	Taurine	δ 3.21 (t, 6.47 Hz), δ 3.41 (t, $J = 6.47$ Hz)	HMDB00251
15	Threonine	δ 1.33 (d, $J = 6.86$ Hz), δ 4.23 (m)	BMSE000859
16	Tyrosine	δ 6.89 (d, $J = 8.5$), δ 7.20 (d, $J = 8.5$)	BML3313

were increased at higher concentrations (2.4 and 3.4 mg/L).

We further treated the embryos with the CB_1 receptor antagonist AM 251 (0.6, 1.2, 2.4 and 4.8 mg/L). The embryos were exposed for 96 h at 24 hpf. The PCA score plot shows a clear separation of the groups treated with AM251 compared to the control group (Fig. 3a). The treated groups were clustered to the negative side of PC1, whereas control samples had positive PC1 scores. This separation was based on 33 % of PC1. The population exposed to higher (2.4 and 4.8 mg/L) and lower (0.6 and 1.2 mg/L) doses of AM251 were separated by PC2 (27 %), having negative and positive PC2 scores, respectively (Fig. 3a). The corresponding loading column plot shows the differentiating metabolites responsible for the separation between treated and control groups (Fig. 3b). The ^1H NMR spectra show the identified signals of the discriminating metabolites (Fig. 4).

In the corresponding loading column plot, most of the signals were shifted towards the PC1 positive side, explaining the lower levels of the related metabolites in treated groups (Fig. 3b). The ^1H NMR signal intensities and shifting of corresponding signals to the positive side in the column loading plot show the clear suppression of lactate, acetate, glutamate, glutamine, choline, glycine, betaine and ATP. The ^1H NMR spectra (Fig. 4) and loading column plot of PC2 (not shown) indicates the increasing levels of phenylalanine and tyrosine in the embryo populations treated with higher doses (2.4 and 4.8 mg/L) of AM251. While lower doses (0.6 and 1.2 mg/L) do not show any significant rise in the production of phenylalanine and tyrosine compared to control group. The relative quantification of important metabolites is shown in supplementary Fig. 1b.

There are adequate data available on the stress and drug driven metabolomics of mammals (Diederich et al. 1997; Milakofsky et al. 1985). This data shows the modulation of amino acid levels in the blood and tissues of rodents and humans mediated by exogenous stimuli. Ethanol is reported to decrease the overall amino acids level in the plasma of rats (Hagman and Eriksson 1990, 1991). L-3,4-dihydrophenylalanine (L-DOPA) is known to significantly inhibit the production of glutamic acid and glutamine in the plasma, heart and brain stem of rats (Diederich et al. 1997). Aspartic acid is decreased in brain stem cells and plasma of rats subjected to L-DOPA and Ethanol, respectively (Diederich et al. 1997, Milakofsky et al. 1989). Here, we report the effects of cannabinoids on the metabolome of zebrafish embryos exposed to Δ^9 -THC and AM251.

Glutamate, choline and glycine are the neurotransmitters involved in the synaptic excitation or inhibition by acting on their respective receptors (Haga et al. 2012; Hollmann and Heinemann 1994, Liu et al. 2010; Lopez-Corcuera et al. 2001). Glutamate mediates the synaptic excitation and inhibition by acting on *N*-methyl-D-aspartate (NMDA)-Sensitive ionotropic glutamate receptors (Hollmann and Heinemann 1994; Liu et al. 2010). A number of Δ^9 -THC regulated activities are mediated by CB_1 receptors distributed throughout the central nervous system and are also present on the presynaptic terminals of glutamatergic synapses (Wiley et al. 2005). Recent studies explicitly show the interaction of cannabinoids also with receptors other than CB_1 and CB_2 modulate the different signal transduction pathways and regulate the neurotransmitter release in brain (Manzanares et al. 2006; Turu and Hunyady 2010). In rodents, several studies have been focused on the effects of cannabinoids to regulate glutamate levels and subsequently

Fig. 3 a Score plot of PCA based on whole range of ^1H NMR signals (δ 0.3– δ 10.0) of zebrafish embryos treated with AM251, 0.6 = 0.6 mg/L AM251; 1.2 = 1.2 mg/L AM251; 2.4 = 2.4 mg/L AM251; 4.8 = 4.8 mg/L AM251 and C = Control (0.01 % Dmsol); **b** Loading column plot of PC1; 1 leucine; 2 lactate; 3 alanine; 4 acetate; 5 glutamine; 6 creatine; 7 choline; 8 glycine; 9 glutamate; 10 betaine, 11 ATP; 12 histidine

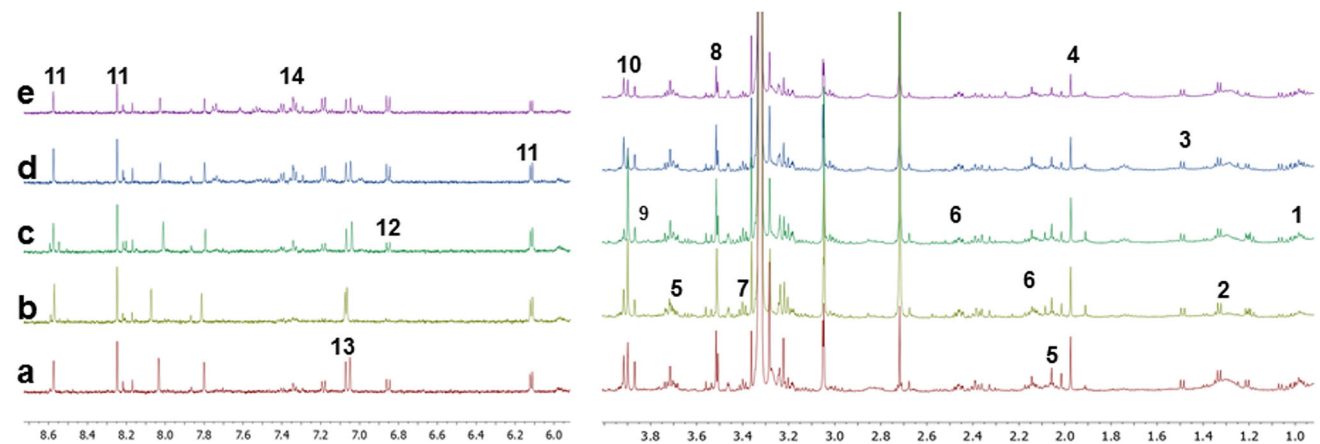
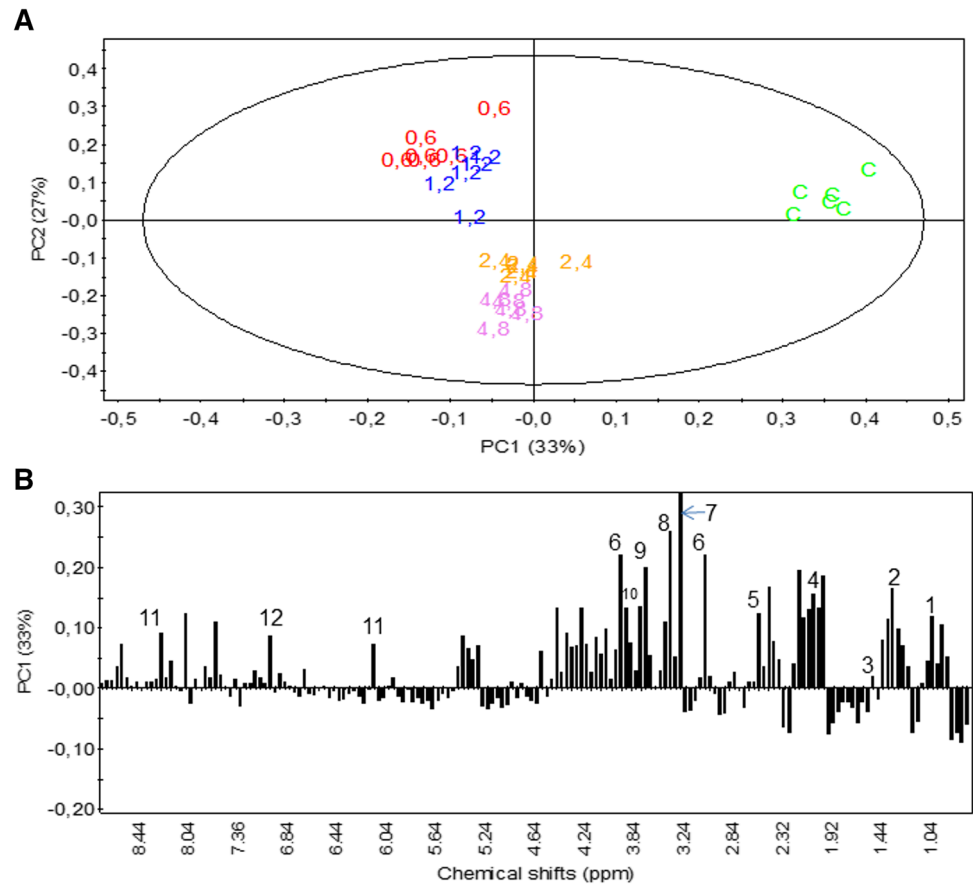


Fig. 4 The ^1H NMR spectra of 5 days old zebrafish embryos raised in **a** 0.01 % DMSO (vehicle) and exposed to **b** 0.6 mg/L of AM251; **c** 1.2 mg/L of AM251; **d** 2.4 mg/L of AM251; **e** 4.8 mg/L of AM251

1 leucine; 2 lactate; 3 alanine; 4 acetate; 5 glutamate; 6 glutamine; 7 taurine; 8 glycine; 9 betaine; 10 creatine; 11 ATP; 12 tyrosine; 13 histidine; 14 phenylalanine

glutamatergic neurotransmission (Antonelli et al. 2004; Castaldo et al. 2007; Ferraro et al. 2001). Δ^9 -THC is known to inhibit glutamatergic neurotransmission (Shen and Thayer 1999; Galanopoulos et al. 2011). In a study of Δ^9 -THC effects on locomotor activity in rats, a higher level of glutamate was found in the rat brain tissues with increased

motor activity at lower dose, while higher dose showed the habituation (Galanopoulos et al. 2011). Previously, Akhtar et al. (2013) have determined the behavioral effects of Δ^9 -THC on the locomotor activity of 5 days old zebrafish embryos. In a chronic exposure (96 h) to Δ^9 -THC and AM251, they reported a significant locomotor stimulation of

zebrafish embryos at lower dose (1.2 mg/L) while higher dose (2.4 mg/L) of Δ^9 -THC showed habituation. Conversely, AM251 caused a significant suppression of locomotor activity. In the current study, Δ^9 -THC induces a dose dependent increase in the levels of glutamate, at doses below LC50 (1.2 and 2.4 mg/L) (Fig. 1a). Exposure to 1.2 mg/L of Δ^9 -THC results in hyper motor activity (Akhtar et al. 2013). In contrast to Δ^9 -THC, AM251 decreases the quantity of glutamate (Fig. 4, supplementary Fig. 1) might be associated with motor suppression (Akhtar et al. 2013). Previous rodents data, present findings and behavior data of zebrafish embryos indicate the important role of CB₁ receptor mediated glutamate regulation in the behavior of zebrafish embryos. The discovery of CB receptors, glutamate receptors and transporters (Gesemann et al. 2010; Rico et al. 2010) in zebrafish embryos and opposite effect of CB₁ agonist and antagonist in current study suggests there might be some interaction of CB₁ receptors and glutamatergic neurotransmission in zebrafish (Tomasini et al. 2002). Overall increase of glutamate in brain tissues or extracellular glutamate levels by Δ^9 -THC might reduce synaptic glutamate levels and consequently affect glutamatergic neurotransmission (Galanopoulos et al. 2011). We have found an overall increase of glutamate levels in embryos treated with Δ^9 -THC. A detail study of glutamate levels in adult or zebrafish embryos brain tissues could provide an insight of Δ^9 -THC and glutamate interaction in zebrafish brain.

Choline is a precursor of acetylcholine (Ach), a compound that mediates cholinergic neurotransmission. Choline is metabolized into betaine, which takes part in the biosynthesis of glycine (Friesen et al. 2007). Glycine is an inducer of glycinergic neurotransmission. Acetylcholine and glycine receptors have been reported in zebrafish embryos (Liu and Westerfield 1992; Rigo and Legendre 2006). In rodents, activation of CB₁ receptors modulates cholinergic, glycinergic and noradrenergic neurotransmission (Szabo and Schlicker 2005). Δ^9 -THC is known to interact with Ach and glycine receptors (Xiong et al. 2011). A higher level of choline and Ach were shown in the five brain regions of mouse exposed to Δ^9 -THC (Tripathi et al. 1987). However, there are also contradictory reports on the Δ^9 -THC modulated effects on Ach release in vivo studies (Acquas et al. 2001; Gessa et al. 1998; Pisanu et al. 2006). Conflicting findings were suggested to be dose-related. Higher doses of CB₁ agonist were proposed to suppress cholinergic transmission while higher doses were reported to enhance it (Tzavara et al. 2003). Δ^9 -THC has also been reported to activate glycine receptors in rodents but there are no studies describing the effects of cannabinoids on the regulation of glycine. In the current study, the visual inspection of ¹H NMR (Fig. 1a) spectra reveals an increase of choline, glycine and betaine in Δ^9 -THC treated embryos.

By contrast, AM251 shows a dose-dependent inhibition of choline and betaine, while a biphasic effect was found in case of glycine (Fig. 4). Although a higher level of choline at an LC₅₀ of Δ^9 -THC (3.4 mg/L) is contradictory to previous findings in rodents, reciprocal effect of antagonist suggests that the CB₁ receptor might also play some role in the regulation of choline and glycine in zebrafish embryos.

Phenylalanine is an essential amino acid and a precursor of tyrosine. Tyrosine hydroxylase (TH) catalyzes the conversion of tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine) a precursor of the neurotransmitter dopamine. The visual analyses of ¹H NMR spectra and relative quantification showed a significant increase in phenylalanine and tyrosine in the embryos exposed to higher concentrations of Δ^9 -THC (2.4 and 3.4 mg/L; Fig. 1a, Supplementary Fig. 1A) and AM251 (2.4 and 4.8 mg/L; Fig. 4, Supplementary Fig. 1B). A number of attempts have been made to determine the functional interaction between cannabinoids and dopaminergic neurotransmission. The presence of CB₁ receptors in TH containing cells is evidence of some connection between cannabinoid and catecholamine synthesis (Bosier et al. 2007). A large number of studies has shown that Δ^9 -THC elevates dopamine levels in the brain, and that this can be blocked by CB₁ receptor antagonists (Le and Goldberg 2005). Δ^9 -THC was reported to increase TH expression in cultured fetal mesencephalic cell line, whereas CB₁ receptor antagonist SR141716A did not show any effect by itself on TH expression but attenuated the activity induced by Δ^9 -THC (Hernandez et al. 1997, 2000). In the present study, a strong correlation between the ¹H NMR signals of tyrosine and phenylalanine indicates a co-regulation of both compounds after exposure to Δ^9 -THC and AM251.

We have recently examined the metabolic effect of solvents (DMSO and ethanol) on zebrafish embryos at 5dpf and found an entirely different embryonic metabolome compared to cannabinoids (data not shown). The previous and current metabolic data suggests that the production of the dopamine precursor tyrosine in cannabinoid-exposed embryos is not a general effect of drugs or solvent toxicity; rather it is a specific effect of cannabinoid exposure on tyrosine regulation. Interestingly, CB₁ receptor agonists and antagonists both increase the synthesis of dopamine precursor which suggests that tyrosine is regulated in a CB₁ receptor-independent manner in the embryos. But the colocalization of CB₁-receptor and TH-expressing cells and modulation of dopamine release in the caudal zone of hypothalamus of zebrafish embryos strengthen the idea of cannabinoid receptor involvement in the regulation of dopaminergic system in zebrafish embryos (Lam et al. 2006). In this study, it is difficult to speculate the impact of cannabinoids on TH activity or an exact role of CB₁ receptors in the regulation of tyrosine or dopaminergic system. Further work is required to confirm the effects of

cannabinoids on TH regulation and subsequently dopaminergic neurotransmission in zebrafish embryos.

In addition to metabolites involved in neurotransmission, several other metabolites were also differentially regulated by Δ^9 -THC and AM251 compared to the control. For example, the higher level of ATP and glucose could reflect higher energy demands in the hyperactive embryos exposed to Δ^9 -THC. A dose-dependent decrease of acetate and a biphasic effect on creatine level was induced by AM251. There is not a straight forward explanation of modulation of these metabolites but these primary metabolites might play an important role in cannabinoid induced effects in zebrafish embryo.

4 Concluding remarks

Our study shows that zebrafish larvae are useful study organisms for performing a whole-organism metabolomics; and that they can be used to study the effects on the metabolome of the intact animal exposed to various compounds. This model may prove useful for examining the changes a drug can elicit in different biosynthetic pathways, by regulating certain metabolites. The results of this study suggest that cannabinoids are involved in the regulation of some of the metabolites involved in neurotransmission in zebrafish embryos.

To the best of our knowledge, there are no available data on the regulation of organic and amino acid levels in the plasma of rats or humans exposed to cannabinoids. Little or nothing is known about the metabolomics of zebrafish larvae exposed to biotic or abiotic stress, therapeutic entities or particularly cannabinoids. Further investigations are needed to confirm the interaction of Δ^9 -THC and different neurotransmitters in specific regions of the zebrafish larval brain.

Recently, ^1H NMR has been used to study the metabolic profiles of adult zebrafish liver (Ong et al. 2009). So, adult zebrafish with complete development could be an alternate for further investigations of brain neuromodulators subjected to different cannabinoids. Techniques like in situ hybridization and Q-PCR could be helpful for the detailed study of enzymes like TH gene expression in different brain regions of adult or larval zebrafish. Further studies with additional tools, and involving different stages of zebrafish development, will substantially increase our understanding of cannabinoid-driven alterations in the neurophysiology of zebrafish embryos. Specifically, these studies will give insights into many important issues including the location and distribution of CB receptors in zebrafish brain, CB receptor subtypes as a target for cannabinoid agonist and antagonist and zebrafish endogenous cannabinoid system.

Compliance with ethical standards

Disclosure Muhammad T. Akhtar designed and conducted the experiment. Muhammad T. Akhtar and Mian Y. Mushtaq analysed the data and also purified the compounds. Muhammad T. Akhtar, Mian Y. Mushtaq and Young H. Choi wrote the article. Robert Verpoorte and Michael K. Richardson provided supervision and research facilities.

Conflicts of interest Authors have no competing interests to declare.

Ethical approval All animal experimental procedures were conducted in accordance with national regulations, which are described in the *Wet op de dierproeven* (article 9) of Dutch Law administered by the Bureau of Animal Experiment Licensing. This national regulation serves as the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EEC, which allows zebrafish embryos to be used up to the moment of free-living (approximately 5-7 dpf).

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