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CHAPTER 6

Metabolic effects of cannabinoids in zebrafish (*Danio rerio*) embryo determined by ^{1}H NMR metabolomics.

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Abstract

Development of efficient high throughput analysis methods is a crucial challenge to speed up the preliminary screening of potential psychostimulants. In this study, we used principal component analysis in combination with ^{1}HNMR to study the metabolic effects of cannabinoid receptor type 1 (CB_1) agonist Δ^9-THC and antagonist AM251in zebrafish embryos. The embryos were exposed at 24 hours post fertilization for 96 hours and analysed at 5dpf. A dose-dependent increase was found in the levels of choline, betaine, glycine, taurine, ATP 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 the higher, lethal dose resulted in abrupt decrease of glutamate levels. In contrast to Δ^9-THC, the antagonist AM251 caused a dose-dependent reduction in betaine, choline, taurine and also inhibited the accumulation of glutamate and glutamine. Interestingly, both compounds induce the production of the dopamine precursor's phenylalanine and tyrosine at higher doses. These findings suggest that the CB_1 receptor is involved in the regulation of metabolites, which are directly involved in neurotransmission in zebrafish embryos. Furthermore, our results show the importance of a ^{1}HNMR based metabolomics platform for the preliminary screening of psychostimulants and pharmaceutical drugs; and in providing a detailed overview of their general effects on the metabolome of a whole organism.

Introduction

Metabolomics is a comprehensive study of a collection of the metabolites present in a cell, a tissue, or an organism at a particular time. 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 (H NMR) (1) is an ideal tool for

the metabolite profiling of biofluids, tissue extracts and intact tissues (Griffin 2004) as well as mass spectrometry. ^{1}HNMR spectroscopy has been used to investigate the biochemical composition of different tissues in an organism (Coen et al. 2003) and drug activity and toxicity assessment in blood serum, liver and testis of rodents (Ekman et al. 2006, Waters et al. 2006) as well as in body fluids such as serum, urine and feces (Le Gall et al. 2011, Mantle et al. 2011). In comparison to other analytical platforms such as mass spectrometry coupled with liquid or gas chromatography, the attractive features of NMR are: simple sample preparation; non-destructive sampling; short measurement time; ease of quantitation and high reproducibility (Kwon et al. 2011)

Many of the Δ^9-THC activities depend on the interaction with cannabinoid receptors (CB) (Lichtman and Martin 1996). Currently, two CB receptors have been identified, CB_1 and CB_2. CB_1 is expressed in brain and periphery, whereas CB_2 is known to be expressed in immune cells and referred as periphery CB_2 receptors (Onaivi et al. 2008). Since the discovery of CB receptors (Matsuda et al. 1990, Munro et al. 1993), a great deal of work has been done to find out their role in cannabinoids mediated activities (Alhamoruni et al. 2012, Fraga et al. 2011, Karmaus et al. 2012). Rodent models are used to study the behavioural 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). These biphasic responses have also been reported in the level of brain monoamines and body temperature (Taylor and Fennessy 1977). Previously, an 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, we used NMR based metabolomics to study the effects of CB receptor type 1 (CB_1) agonist Δ^9-THC

and CB_1-antagonist AM251 on metabolic profile of 5 days old post fertilization (5 dpf) zebrafish embryos.

Materials and methods

Ethics statement

All animal experimental procedures were conducted in accordance with national regulations, 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).

Zebrafish handling

Male and female adult zebrafish (*Danio rerio)* of wild type were purchased from Selecta Aquarium Speciaalzaak (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 ^{o}C and 23 ^{o}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, Leiden, The Netherlands).

Defined embryo buffer

Ten percentage (v/v) of Hank's balanced salt solution 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 (Irons et al. 2010; Macphail et al. 2009).

Embryo care

Eggs were obtained by random pairwise 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 hours 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.

Preliminary range-finding

To determine a suitable range of concentrations of testing compounds, we performed range-finding experiments. 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 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.

Refined geometric series and LC50 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 AM251 was 0.3-9.6 mg/L 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, USA).

Embryo treatment and collection

One hundred and twenty embryos per replicate were collected. After 24 hours the embryo 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 falcon tube. The collected embryos were immediately frozen in liquid nitrogen and stored at -80 $^{\circ}$C till further analysis.

Sample preparation and Extraction

A fairly simple sample preparation method already was used for plant metabolomics (Abdel-Farid et al. 2009, Ali Kashif et al. 2010, Jahangir et al. 2008, Kim et al. 2010a, Kim et al. 2010b). Embryos were freeze dried and transferred to a micro tubes (2 ml) to which 1ml of 50% CH_3OH-*d4* in D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.01% TMSP (w/w) was added. The

mixture was vortexes for one min, sonicated for 20 min, and centrifuged at 13,000 rpm at room temperature for ten minutes. An aliquot (650 µL) was then transferred to a 5mm-NMR tube. NMR spectra were recorded at 25°C on a 500MHz 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 requiring with the parameters as used by our group (Kim et al. 2010b).

Data Pre Processing and Analysis

Spectral 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. PCA was performed with the SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) based on a Pareto scaling method. All of the spectra were referenced, base line, 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).

Results

Principal component analysis

Principal component analysis (PCA) is a statistical tool applied to process a huge number of data sets by reducing the multidimensionality of the data without losing required information. This technique helps to assess the possible differences and similarities between the samples. In order to look into overview of metabolic changes firstly we applied PCA to binned ^1H NMR variables representing the key metabolites regulated differently in the Δ^9-THC treated and non-treated zebrafish embryos. The PCA revealed that four components were

enough to represent 84% of the variation in the data. The score plot shows that 40.6% of the separation is based on PC1 and 21.8% is based on PC2 scores (Fig 1B). Inspection of the loading plot reveals that the major source of variability among the samples is strongly correlated with the dose of Δ^9-THC (Fig 1C). The control (HBSS) and vehicle (HBSS with 0.01% DMSO) groups tend to cluster together along the positive side of PC1 (horizontal line), while the most affected group or best separated group from the control is the medium dose group (1.2mg/L). The high dose (2.4 and 3.4 mg/l) groups cluster together in between low and medium dose (Fig 1B).

PLS modeling with Orthogonal signal correction

The NMR data was subjected to Partial least square data analysis (PLS-DA). A 'Y' variable was included in the data representing different classes (Control, 0.01% DMSO treated, Low dose (0.6mg/L Δ^9-THC), medium dose (1.2mg/L Δ^9-THC), high dose (2.4mg/L Δ^9-THC) and LC50 (3.4mg/L Δ^9-THC), while each class was given a specific value. The objective of PLS-DA modeling is to relate two data matrices i.e X and Y to each other by a linear multivariate model (Wold et al. 2001). Each class was assigned a different value as a Y variable. After cross validation, the model showed three components, which were enough to describe 57% of the variation in data.

The 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 the score plot. To remove these uncorrelated data we developed a model with the use of a data filter called orthogonal signal correction (OSC). This filter had already been applied successfully in several studies (Gavaghan et al. 2002, Hauksson et al. 2001). The filter calculates a vector which is

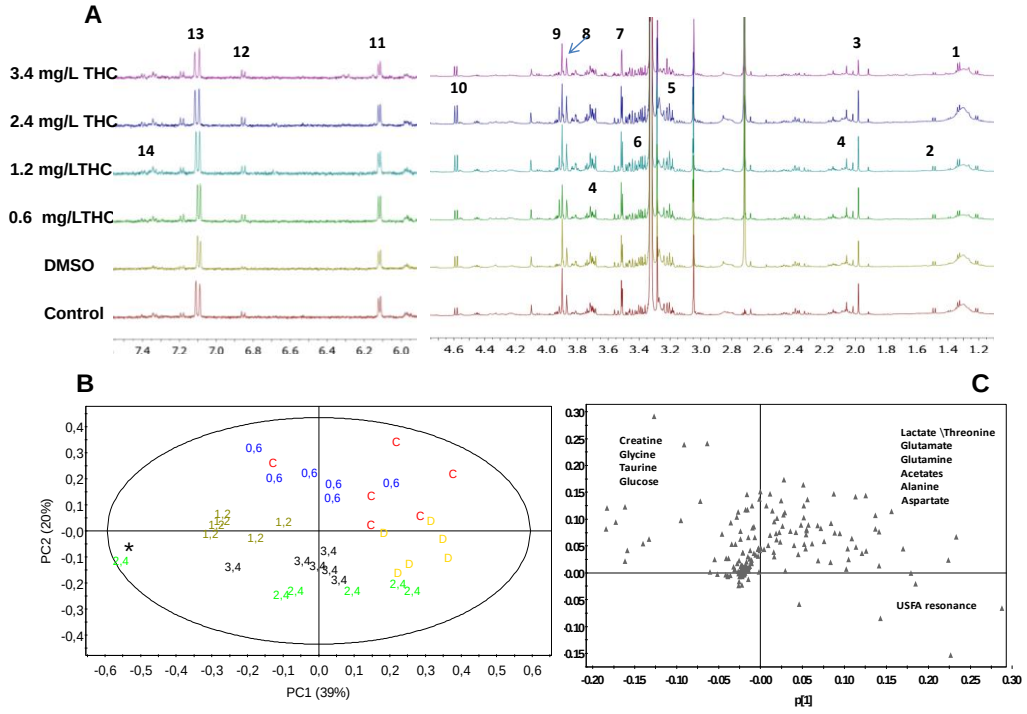

Figure 1. **A**; Comparison of ^{1}H NMR spectra of zebrafish embryos, showing the region of interest after treating the embryos with Control, 0.01% DMSO and 0.6, 1.2, 2.4 and 3.4 mg/L of Δ^{9}-THC. (1) Lactate (2) Alanine, (3) Acetate, (4) Glutamate (5) Choline, (6) Taurine, (7) Glycine, (8) Betaine, (9) Creatine, (10) Glucose, (11) ATP, (12) Tyrsoine, (13) Histidine, (14) Phenyalanine. **B**; Scatter plot from Principal component analysis. (C) Control, (D) 0.01% DMSO, (0.6) 0.6 mg/L Δ^{9}-THC , (1.2) 1.2 mg/L Δ^{9}-THC, (2.4) 2.4 mg/L Δ^{9}-THC, (3.4) 3.4 Δ^{9}-THC; * represent the outlier. **C;** Representative loading plot of PCA analysis.

orthogonal to Y and then by multiplying this vector to the loadings (represent the orientation of the model in the space) subtract it from X-data. This process removes the un-correlated data; the residual matrix left is the representative matrix of the variables responsible for the variation in the model. The filter was used before applying any model. After filtering, the data was subjected to partial least square analysis. 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 by using the permutation test with 100 permutations as shown in Fig 2C. 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 (2001). The scatter plot from this data shows the grouping of control, DMSO and low dose (0.6mg/L) treated groups on the negative side of PLS-DA1, while the moderate dose (1.2 mg/L) clustered on positive side of component 1 and negative side of component 2 (Fig 2A). The highest dose (3.4mg/L) group, on the other hand, belongs to the positive side of both component 1 and component 2.

S-Plot

The corresponding loading plot or S-plot shows the variables affecting the separation in the scatter plot (Fig 2B). The variables (^{1}H NMR-chemical shifts) related to certain key metabolites were identified and are shown in Chapter 7, Table 1. The identification of metabolites confirm that the levels of amino acids such as glutamate, glutamine, isoleucine, alanine, threonine, aspartate, taurine, phenylalanine, choline, creatine and glycine are affected in embryo populations 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 highest dose (3.4 mg/L) of Δ^9-THC. The levels of phenylalanine, taurine and choline were increased in the samples treated with higher doses of Δ^9-THC compared to the control group. Visual inspection of ^{1}H NMR spectra reveals that different doses of Δ^9-THC resulted in differential changes in the levels of metabolites in the embryo population (Fig 1A). The levels of glucose, glutamate, acetate and adenosine triphosphate (ATP) increased with the increasing dose of Δ^9-THC (0.6 to 2.4

mg/L of Δ^9-THC) and decreased at the highest dose of Δ^9-THC (3.4 mg/L). A dose dependent increase was observed in the level of choline, glycine and

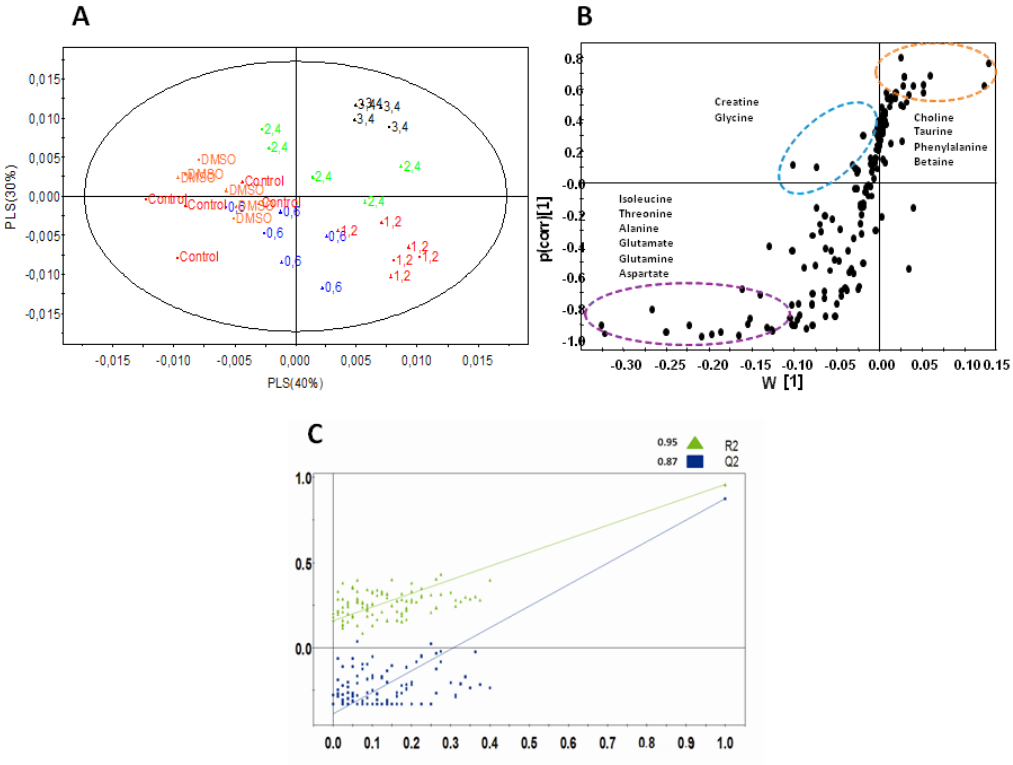

Figure 2. A; Scatter plot from Partial least square analysis with orthogonal signal correction filter. Figure shows the embryos treated with Control; 0.01% DMSO; 0.6, 1.2, 2.4 and 3.4 mg/L of Δ^9-THC. * represent the outlier. **B**; S-Plot shows the markers (variables) responsible for the separation. Upper right corner correspond to the high doses of Δ^9-THC while lower left corner correspond with the lower doses and control group of Δ^9-THC. **C;** Cross validation of two components. **C;** validation of PLS-DA model with permutation test (100 permutations).

betaine. Phenylalanine and tyrosine levels were increased at higher doses (2.4 and 3.4 mg/L).

In additional experiments, we exposed embryos to the CB_1 receptor antagonist AM251 (0.6, 1.2, 2.4 and 4.8 mg/L). The 24 hpf embryos were exposed for 96 h to the test compounds. The PCA score plot shows a clear separation of the groups treated with AM251 from the control group (Fig 3A). The treated groups were clustered to the negative side of PC1, whereas control samples had a positive PC1 score. 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 ^{1}H NMR shows the signals of the discriminating metabolites identified (Fig 4).

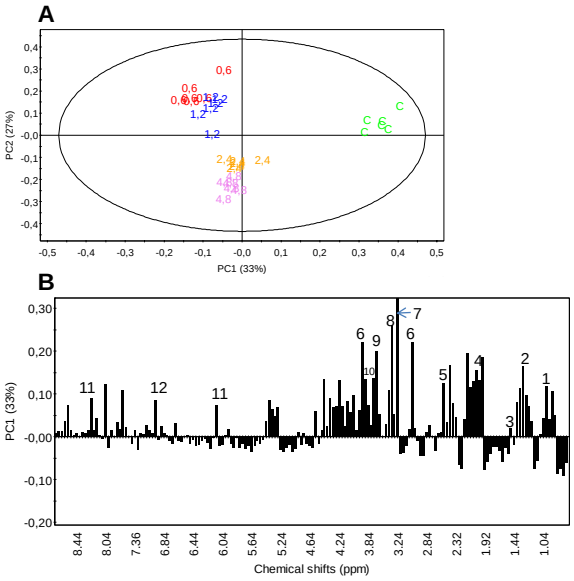

Figure 3. A, Score plot (PC1 vs PC2) of PCA based on whole range of ^{1}H NMR signals (δ 0.3- δ 10.0) of zebrafish embryos treated with AM 251, 0.6= 0.6 mg/L AM251; 1.2= 1.2 mg/L AM251; 2.4= 2.4 mg/L AM251; 4.8= 4.8 mg/L AM251 and C= Control (0.01% Dmso); **B**, Loading column plot of PC1. 1, leucine; 2, lactate;

3,alanine; 4, acetate; 5 glutamine; 6, creatine; 7, choline;
8, glycine; 9, glutamate; 10, betaine, 11; ATP; 12, histidine.

In the corresponding loading column plot, most of the signals are found at the PC1 positive side, corresponding with lower levels of the related metabolites in treated groups. From the ^{1}H NMR signals at positive side in column loading plot belongs to lactate, acetate, glutamate, glutamine, choline, glycine, betaine and ATP, which therefore all present at lower levels in the treated embryos. The ^{1}H NMR spectra (Fig 4) and loading column plot of PC2 (not shown) show increasing levels of phenylalanine and tyrosine in the embryo populations treated with higher doses (2.4 and 4.8 mg/L) of AM251. Lower doses (0.6 and 1.2 mg/L) do not show any significant rise in phenylalanine and tyrosine levels compared to the control group.

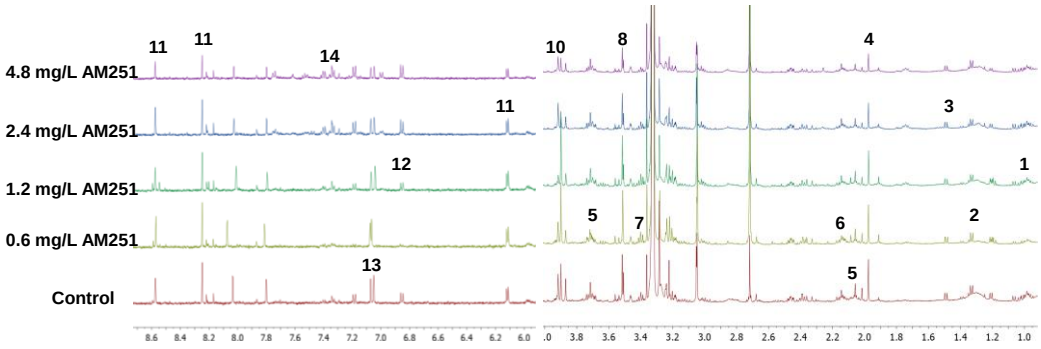

Figure 4. The ^{1}H NMR spectra of 5 days old zebrafish embryos raised in 0.01% DMSO (Control) and exposed to 0.6, 1.2, 2.4 and 4.8 mg/L of AM251. 1, leucine; 2, lactate; 3, alanine; 4, acetate; 5, glutamate; 6, glutamine; 7, taurine; 8, glycine; 9, betaine; 10, creatine; 11, ATP; 12, tyrosine; 13, histidine; 14, phenylalanine.

Discussion

There is an extensive dataset available on the stress and drug-driven metabolome of mammals (Diederich et al. 1997, Milakofsky et al. 1985). These data show 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 acid level in the plasma of rats (Hagman and Eriksson 1990, 1991). 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, for first time, we report the effects of cannabinoids on the metabolome of zebrafish embryos exposed to Δ^9-THC and AM251.

Glutamate, choline and glycine are neurotransmitters involved in synaptic excitation or inhibition by acting on their respective receptors (Haga et al. 2012, Hollmann and Heinemann 1994, Liu J. et al. 2010, Lopez-Corcuera et al. 2001). Glutamate mediates synaptic excitation and inhibition by acting on N-methyl-D-aspartate (NMDA)-sensitive ionotropic glutamate receptors (Hollmann and Heinemann 1994, Liu J. et al. 2010). A number of Δ^9-THC regulated activities are mediated by cannabinoid receptors of the CB_1 class distributed throughout the central nervous system and are also present on the presynaptic terminals of glutamatergic synapses (Wiley et al. 2005).

In rodents, several studies have been focused on the effects of cannabinoids on the regulation of glutamate levels and subsequent glutamatergic neurotransmission (Antonelli et al. 2004, Castaldo et al. 2007, Ferraro et al. 2001). In one study, Δ^9-THC was shown to inhibit glutamatergic neurotransmission (Shen and Thayer 1999, Galanopoulos et al. 2011). In a study of Δ^9-THC effect 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 doses showed habituation (Galanopoulos et al. 2011).

Previously, we determined the behavioral effects of Δ^9-THC on the locomotor activity of 5 day old zebrafish embryos (Thesis chapter 5). In a chronic exposure (96 hours) to Δ^9-THC and AM251, we found a significant locomotor stimulation of zebrafish embryos at the lower dose (1.2 mg/L) while a higher dose (2.4 mg/L) of Δ^9-THC caused habituation. Conversely, AM251 caused a significant suppression of locomotor activity. In the current study, Δ^9-THC induced a dose dependent increase in the levels of glutamate at doses below LC_{50} (1.2 mg/L and 2.4 mg/L) (Fig 1A). Exposure to 1.2 mg/L of Δ^9-THC results in locomotor hyperactivity (Thesis Chapter 5). In contrast to Δ^9-THC, AM251 produced a dose-dependent decrease in the quantity of glutamate (Fig 4) associated with locomotor suppression (Thesis chapter 5).

Previous data from rodent studies, together with present findings and our previous behavioural study, indicate an important role of CB_1 receptor mediated glutamate regulation in the behavior of zebrafish embryos. The discovery of CB receptors and glutamate receptors and transporters (Gesemann et al. 2010, Rico et al. 2010) in zebrafish embryos, and the opposite effect of CB_1 agonist and antagonist in the current study, together suggest a strong interaction of CB_1 receptors and glutamatergic neurotransmission in zebrafish (Tomasini et al. 2002).

It has been suggested that an overall increase of glutamate levels induced in brain tissues 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 whole embryos treated with Δ^9-THC. Therefore, in order to investigate this question further, a detailed study of glutamate levels in zebrafish brain tissue could provide an insight into Δ^9-THC and glutamate interaction in the zebrafish brain.

The effects identified here on choline levels are significant because choline is the precursor of the neurotransmitter acetylcholine (ACh) which mediates cholinergic neurotransmission. Choline is metabolized into betaine which takes part in the biosynthesis of glycine (Friesen et al. 2007). Glycine is a mediator of glycinergic neurotransmission. ACh and glycine receptors have been reported in zebrafish embryos (Liu and Westerfield 1992, Rigo and Legendre 2006). In rodents, activation of CB 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).

Higher levels of choline and ACh have been found in the five brain regions of mice 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 *in vivo* studies (Acquas et al. 2001, Gessa et al. 1998, Pisanu et al. 2006). Subsequently, these conflicting findings were attributed to different doses of Δ^9-THC used in the different studies (Solinas et al. 2007). Tzavara et al. (2003) reported a biphasic effect of CB_1 agonist on the modulation of Ach release and proposed that higher doses of CB_1 agonists act as an ACh depressant and lower doses act as a stimulator of ACh neurotransmission. Δ^9-THC was also reported to activate glycine receptors in rodents, but there are no studies describing the effects of cannabinoids on the regulation of glycine. The visual analyses of ^{1}H NMR spectra reveal a dose dependent increase of choline, glycine and betaine in Δ^9-THC treated embryos (Fig 1A). On the contrary, AM251 shows a dose dependent inhibition of choline and betaine, while a biphasic effect was found in case of glycine (Fig 4). Although increase in choline at the LC_{50} value of Δ^9-THC (3.4 mg/L) is contradictory to previous findings in rodents, the reciprocal effect of the antagonist suggests that CB_1 might also play some role in the regulation of choline and glycine levels in zebrafish embryos.

Another metabolite whose levels were found to vary significantly between treatments in our study was phenylalanine. This is an essential amino acid and a precursor of tyrosine. Tyrosine hydroxylase (TH) converts tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine) a precursor of the neurotransmitter dopamine. Visual inspection of ^{1}H NMR spectra showed a significant increase of phenylalanine and tyrosine levels in the embryos exposed to higher doses of Δ^{9}-THC (2.4 and 3.4 mg/L) and AM251 (2.4 and 4.8 mg/L) (Fig 1A, Fig 4).

A number of attempts have been made to determine the functional interaction between cannabinoids and dopamine neurotransmission. The presence of CB_1 receptors in TH containing cells is evidence of some connection between cannabinoids and catecholamine synthesis (Bosier et al. 2007). Many studies have shown that Δ^{9}-THC elevates dopamine levels in the brain, and that this effect can be blocked by a CB_1 receptor antagonist (Le Foll and Goldberg 2005). Δ^{9}-THC was reported to increase the TH expression in a cultured fetal mesencephalic cell line; by contrast, the CB_1 receptor antagonist SR141716A had no such effect on TH expression. It did, however, attenuate the effect of Δ^{9}-THC on TH expression (Hernandez et al. 2000, Hernandez et al. 1997). The strong correlation found in the present study between the ^{1}H NMR signals of tyrosine and phenyalanine suggests a co-regulation of both compounds after exposure to Δ^{9}-THC and AM251.

An important question is the specificity of the metabolomic patterns induced by Δ^{9}-THC and AM251 in this study. That is, whether similar might be due to any compound exposure. This seems unlikely because we have recently examined the metabolic effect of solvents (DMSO and ethanol) on the 5 days old zebrafish embryos and found that they induce a entirely different metabolic profile compared to that found here for cannabinoids (Thesis chapter 7). The previous and current metabolic data suggest that the accumulation of the

dopamine precursor (tyrosine) in cannabinoid administered embryos is not a general effect of drugs or solvent toxicity rather it is a specific effect of cannabinoid exposure on tyrosine regulation.

Interestingly, both the CB_1 receptor agonist and antagonist used here increased the levels of the dopamine precursor phenylalanine; this suggests that tyrosine might be regulated in a CB_1 receptor-independent manner in the embryos. Lam et al. 2006 found that CB_1-receptors and TH-expressing cells were co-localized in the caudal zone of the hypothalamus of zebrafish embryos. Together, these findings strengthen the possibility of cannabinoid receptor involvement in the regulation of the dopaminergic system in zebrafish embryos.

Based on the current data, it is not possible to draw conclusions on the impact of cannabinoids on TH activity, or on the exact role of CB_1 receptors in the regulation of tyrosine synthesis or of the dopaminergic system. Therefore, further molecular and functional studies are required to confirm the effects of cannabinoids on TH regulation and subsequently dopaminergic neurotranmission in zebrafish.

In addition to neurotransmitters, several other metabolites were also differentially regulated by Δ^9-THC and AM251 compared to controls. For example, the higher level of ATP and glucose might indicate a higher energy demand of the hyperactive embryos exposed to Δ^9-THC. A dose dependent decrease of acetate and a biphasic effect on creatine levels was induced by AM251. There is no straightforward explanation of the modulation of these metabolites, but it is at least possible that they might play some role in cannabinoid induced effects in zebrafish embryo.

Our study shows that zebrafish larvae are a useful model for performing metabolomic profiling in a whole animal exposed to pharmacological agents. This approach may help in identifying the changes a drug could elicit in

different biosynthetic pathways. The result of our study shows that cannabinoids are involved in the regulation of metabolites which are directly involved in neurotransmission in zebrafish embryos. Further investigations are needed to characterize the interactions of Δ^9-THC with different neurotransmitter pathways in different regions of the zebrafish brain. Recently, 1H NMR was used to study the metabolic profile of adult zebrafish liver (Ong et al. 2009). Meatbolomic studies need to be complemented by the use of techniques such as in situ hybridization, immunochemistry and Q-PCR in order to gain a complete picture of gene expression, protein distribution and metabolite profiles. Questions that remain to be answered are the distribution of CB receptors in the adult zebrafish brain, the differential roles of cannabinoid receptor subtypes, and the function of the zebrafish endogenous cannabinoid system.

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