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Themed Section: Cannabinoids in Biology and Medicine, Part II

RESEARCH PAPER

Differential transcriptional profiles mediated by exposure to the cannabinoids cannabidiol and Δ^9-tetrahydrocannabinol in BV-2 microglial cells

Ana Juknat[1]*, Maciej Pietr[2]*†, Ewa Kozela[1]*, Neta Rimmerman[2], Rivka Levy[2], Giovanni Coppola[3], Daniel Geschwind[3] and Zvi Vogel[1,2]

[1]*The Dr. Miriam and Sheldon G. Adelson Center for the Biology of Addictive Diseases, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel,* [2]*Neurobiology Department, Weizmann Institute of Science, Rehovot, Israel, and* [3]*Program in Neurogenetics, Department of Neurology, University of California, Los Angeles, CA, USA*

Correspondence
Zvi Vogel, The Dr. Miriam and Sheldon G. Adelson Center for the Biology of Addictive Diseases, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel. E-mail: zvi.vogel@weizmann.ac.il

*These authors contributed equally to this work.

†Deceased August 2009.

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BACKGROUND AND PURPOSE

Apart from their effects on mood and reward, cannabinoids exert beneficial actions such as neuroprotection and attenuation of inflammation. The immunosuppressive activity of cannabinoids has been well established. However, the underlying mechanisms are largely unknown. We previously showed that the psychoactive cannabinoid Δ^9-tetrahydrocannabinol (THC) and the non-psychoactive cannabidiol (CBD) differ in their anti-inflammatory signalling pathways.

EXPERIMENTAL APPROACH

To characterize the transcriptional effects of CBD and THC, we treated BV-2 microglial cells with these compounds and performed comparative microarray analysis using the Illumina MouseRef-8 BeadChip platform. Ingenuity Pathway Analysis was performed to identify functional subsets of genes and networks regulated by CBD and/or THC.

KEY RESULTS

Overall, CBD altered the expression of many more genes; from the 1298 transcripts found to be differentially regulated by the treatments, 680 gene probe sets were up-regulated by CBD and 58 by THC, and 524 gene products were down-regulated by CBD and only 36 by THC. CBD-specific gene expression profile showed changes associated with oxidative stress and glutathione depletion, normally occurring under nutrient limiting conditions or proteasome inhibition and involving the GCN2/eIF2α/p8/ATF4/CHOP-TRIB3 pathway. Furthermore, CBD-stimulated genes were shown to be controlled by nuclear factors known to be involved in the regulation of stress response and inflammation, mainly via the (EpRE/ARE)-Nrf2/ATF4 system and the Nrf2/Hmox1 axis.

CONCLUSIONS AND IMPLICATIONS

These observations indicated that CBD, but much less than THC, induced a cellular stress response in microglial cells and suggested that this effect could underlie its anti-inflammatory activity.

LINKED ARTICLES

This article is part of a themed section on Cannabinoids in Biology and Medicine. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2012.165.issue-8. To view Part I of Cannabinoids in Biology and Medicine visit http://dx.doi.org/10.1111/bph.2011.163.issue-7

Abbreviations

CBD, cannabidiol; GSH, glutathione; Nrf2, nuclear factor-erythroid 2-related factor 2; THC, Δ^9-tetrahydrocannabinol

Introduction

Preparations derived from *Cannabis sativa* (marijuana and hashish) are recognized nowadays as potentially addictive, as well as having wide medical applications (see Earleywine, 2002; Kogan and Mechoulam, 2007; Pertwee, 2009). Therapeutic uses of marijuana and its active constituents, the cannabinoids, range from treatment of nausea, vomiting and cachexia (in cancer chemotherapy and AIDS patients), to handling of chronic inflammatory pain, glaucoma, epileptic seizures, Parkinsonian tremor as well as multiple sclerosis (see Pertwee, 2002; Guzman, 2003; Di Marzo and De Petrocellis, 2006; Kogan and Mechoulam, 2007). Cannabinoids act as potent immunosuppressive and anti-inflammatory agents and have been reported to mediate modulatory activities on immune cell functions (Klein *et al.*, 1998; McKallip *et al.*, 2002; Cabral and Staab, 2005; Klein and Cabral, 2006; Kozela *et al.*, 2010; Rieder *et al.*, 2010). In addition, cannabinoids have pro-apoptotic, neuroprotective and anti-tumour properties (van der Stelt and Di Marzo, 2005; Massi *et al.*, 2006; Galve-Roperh *et al.*, 2000).

Two cannabinoid receptors have been characterized so far. The cannabinoid CB_1 receptor, which is mainly present in neural cells, mediates the psychoactive and addictive activities of cannabinoids, and the CB_2 receptor, which is expressed mainly in the immune system, is involved in cannabinoid immunomodulation (receptor nomenclature follows Alexander *et al.*, 2011). The major psychoactive component of marijuana, Δ^9-tetrahydrocannabinol (THC) is equally effective at either of these receptors (Rhee *et al.*, 1997) and has effects on both the immune and the nervous systems (Cabral and Staab, 2005; Le Foll and Goldberg, 2005; Cabral and Griffin-Thomas, 2008; 2009; Woelkart *et al.*, 2008).

One of the current pharmacological challenges is to elucidate the mechanisms underlying the beneficial properties ascribed to marijuana in order to develop cannabinoid-based therapeutics lacking the adverse psychotropic effects. Thus, another compound abundant in *C. sativa* extracts, cannabidiol (CBD), is under extensive investigation (Mechoulam *et al.*, 2002, 2007; Pertwee, 2005; Izzo *et al.*, 2009). CBD exhibits anti-inflammatory, antioxidant and neuroprotective properties, but, unlike THC, is devoid of psychotropic effects and has very low affinity for both CB_1 and CB_2 receptors (see Mechoulam *et al.*, 2002, 2007; Izzo *et al.*, 2009).

Cell-type specific induction of cell death is considered to be one of the primary actions of many plant-derived immunosuppressants and anticancer drugs (Ho and Lai, 2004; Fesik, 2005). Early investigations have postulated a role for cell death in the regulation of immune function by cannabinoids (Raz and Goldman, 1976; Davies *et al.*, 1979). Both THC and CBD have been shown to induce apoptosis in leukaemia and primary lymphocytes (McKallip *et al.*, 2002, 2006; Gallily *et al.*, 2003; Do *et al.*, 2004; Wu *et al.*, 2008; Lee *et al.*, 2008a). In addition, THC has been shown to induce apoptosis in macrophages, dendrocytes and glioma cells (Zhu *et al.*, 1998; McKallip *et al.*, 2002; Goncharov *et al.*, 2005). Although the mechanisms by which these cannabinoids trigger apoptosis are not fully understood, a common feature of CBD and THC treatments in transformed and primary lymphocytes is the elevation of intracellular reactive oxygen species (ROS) accompanied by glutathione (GSH) depletion (McKallip *et al.*, 2006; Wu *et al.*, 2008; Lee *et al.*, 2008a). Importantly, in U87MG human astrocytoma and in C6 glioma cells, induction of cell death by THC was reported to be associated with stress-related gene expression with a central role for the p8-ATF4-TRB3 pathway (Carracedo *et al.*, 2006a,b; Salazar *et al.*, 2009).

We have previously shown that CBD and THC have different effects on anti-inflammatory pathways in lipopolysaccharide (LPS)-treated BV-2 cells. CBD reduces the activity of the NF-κB pathway and up-regulates the activation of the STAT3 transcription factor. However, both CBD and THC decrease the activation of the LPS-induced STAT1 transcription factor, a key player in IFNβ-dependent pro-inflammatory processes (Kozela *et al.*, 2010). Therefore, we decided to characterize the transcriptional effects of CBD and THC in surveillant (resting) BV-2 microglial cells. BV-2 cells exhibit morphological, phenotypic and functional properties associated with freshly isolated microglial cells (Blasi *et al.*, 1990; Bocchini *et al.*, 1992; Ulrich *et al.*, 2001; Kim *et al.*, 2004). Although BV-2 cells are used as a model of microglial cells, Horvath *et al.* (2008) and Pietr *et al.* (2009) reported that BV-2 cells did not exactly model the response of microglial cells in primary culture, when the reactive and inflammatory profiles of these cell cultures were compared, for instance, following treatment with LPS or IFNγ.

We have performed comparative gene profiling analyses of gene expression in BV-2 cells treated with either CBD or THC. The results of this study showed that CBD exerted a much greater effect on gene expression compared with that of THC. From the 1298 transcripts found to be differentially regulated by CBD and/or THC, 1204 gene probe sets were regulated by CBD and only 94 by THC. The Ingenuity Pathway Analysis (IPA) linked the CBD effects to activation of stress- and cell death-related signalling pathways, showing that CBD, and to a much lesser extent THC, activated the (EpRE/ARE)-Nrf2/ATF4 system and the GCN2/eIF2α/p8/ATF4/CHOP-TRIB3 pathway, known to be leading to autophagy and apoptotic cell death.

Methods

Microglial cell culture

The immortalized murine BV-2 microglial cell line was kindly provided by Prof. E.J. Choi from Korea University (Seoul, Korea). BV-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) containing 4.5 $g \cdot L^{-1}$ glucose, supplemented with 5% fetal calf serum, penicillin (100 $U \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$) (Biological Industries Ltd, Kibbutz Beit Haemek, Israel), under a humidified 5% CO_2 atmosphere at 37°C. Cells were treated with either THC or CBD (both at 10 μM) for 6 h, unless otherwise indicated.

Total RNA extraction

Total RNA was obtained using the PerfectPure RNA extraction kit (5Prime, Darmstadt, Germany) following the manufacture's instructions. Quantification of extracted RNA was performed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA integ-

rity was assessed by electrophoresis on ethidium bromide-stained 1% agarose gels. Purity of the RNA was analysed using the Agilent Bioanalyzer Nanochips (Agilent Technologies, Palo Alto, CA, USA).

Microarray transcript analysis

Total RNA (200 ng) was amplified and labelled, and then hybridized onto Illumina MouseRef-8 v1.1 Expression BeadChip (Illumina Inc., San Diego, CA, USA), querying the expression of >24 000 RefSeq-curated gene targets and 822 random sequences used for the assessment of background noise. Six independent preparations of mRNA were analysed on six independent BeadChips for each of the treatment conditions. Arrays were processed and scanned with Illumina BeadStation platform according to the manufacturer's protocol. Raw data were analysed using the Bioconductor packages (http://www.bioconductor.org; Gentleman *et al.*, 2004). Quality-control analysis was performed using the inter-array Pearson correlation and clustering based on variance. Raw data were log2 transformed and normalized using quantile normalization. Analysis of differential expression was performed using a linear model fitting (LIMMA package, Smyth, 2005). Differentially expressed genes were classified according to their gene ontology (GO), using Bioconductor packages and online tools [Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources, http://david.abcc.ncifcrf.gov/; Huang *et al.*, 2009; and WebGestalt, http://bioinfo.vanderbilt.edu/webgestalt/]. Significantly over-represented GO categories were defined using a threshold for statistical significance set at $P < 0.05$. Literature data mining for co-occurrence of gene names and keywords of interest (e.g. oxidative stress, mitochondria) was performed using Chilibot (http://www.chilibot.net/) search. Cellular pathway association was analysed according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/).

Ingenuity pathway analysis

Pathway and global functional analyses were performed using IPA 6.0 (Ingenuity® Systems, http://www.ingenuity.com/). A data set containing gene identifiers and corresponding expression values was uploaded into the application, and each gene identifier was mapped to its corresponding gene object using the Ingenuity Pathways Knowledge Base (IPKB). The functional and canonical pathways' analyses identified the biological functions and the pathways from the IPA library that were most significant to the data set. Genes from the data set that met the *P*-value cut-off of 0.005 and were associated with biological functions or with a canonical pathway in the IPKB were considered for analysis. Fisher's exact test was used to calculate a *P*-value determining the probability that each biological function and/or canonical pathway assigned to this data set is not due to chance alone.

Primer design for quantitative real time PCR validation assays

Primer sets for a selected collection of transcripts used in quantitative real time reverse transcription polymerase chain reaction (qPCR) validation were designed using the computer program Primer Express (Perkin Elmer/Applied Biosystems, Foster City, CA, USA) or Primer Quest, an online tool provided by Integrated DNA Technologies (http://test.idtdna.com) (Table 1). Wherever possible, designs with at least one of the primer sequences located on an intron–exon boundary were chosen, to avoid co-amplification of minor amounts of genomic DNA that could be present in the RNA samples. All primers were analysed using nucleotide BLAST to ensure primer specificity for the gene of interest (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

qPCR analysis

cDNA was generated by the QuantiTect Reverse Transcription kit containing gDNA 'wipe out' (to eliminate contamination with genomic DNA), according to the manufacturer's instructions (Qiagen, AG, Basel, Switzerland). qPCR was carried out in 0.1 mL tubes in the Rotor-Gene 3000 instrument (Corbett Research, Sydney, Australia). PCR reaction mixtures (20 μL) contained cDNA samples (in 3 μL), 5 pmol of each of the two primers and 10 μL of Blue SYBR Green PCR Rox Mix containing the DNA polymerase (Abgene House, Epsom, Surrey, UK) as detailed by Butovsky *et al.* (2006). For each of the examined mRNAs, normal and mock reverse transcribed samples, as well as no template control (total mix without cDNA) were run. In order to obtain the dilution curves for PCR amplification efficiencies, each set of duplicate PCR reactions was performed with five different concentrations of each of the cDNAs tested. The PCR reactions were subjected to the following conditions: 15 min at 95°C to activate the Termo-start DNA polymerase present in the PCR Master Mix, followed by 40 cycles consisting of: 15 s at 94°C, 30 s at 60°C and 30 s at 72°C. Fluorescence was measured at the end of each elongation step. A melting curve was generated at the end of each run to ensure product uniformity and to rule out primer–dimers and presence of splice variants. For each sample, an amplification plot was generated, showing the increase in the SYBR green fluorescence for each cycle of PCR. A threshold cycle value *Ct* was calculated from the exponential phase of each PCR sample and a standard curve for each gene was plotted (*Ct* vs. log DNA concentration). For each sample, the expression level of the gene of interest was normalized to the reference gene, β_2-microglobulin (*B2m*), whose expression was found not to be affected by the various treatments. RNA expression levels are expressed as fold change using the calculation method described by Pfaffl (2001). The qPCR experiments were repeated four times using different mRNA batches from independent experiments and reactions were performed in duplicates for each cDNA sample.

Statistical analysis

qPCR data were plotted as the mean ± SEM of three or more independent experiments. Statistical significance was assessed using a one-way ANOVA and followed by Bonferroni *post hoc* multiple comparison test as implemented in the version 6.1 (R2007b) Statistics Toolbox Software, MATLAB, MathWorks (http://www.mathworks.com/help/toolbox/stats/rn/brasjn_.html). A *P*-value < 0.05 was defined as statistically significant.

Table 1

Primer sequences used for qPCR

		Sequence (5′ → 3′)	Length (bp)[a]	Accession[b]
B2mg	FW	ATG GGA AGC CGA ACA TAC TG	176	NM_009735.3
	RV	CAG TCT CAG TGG GGG TGA AT		
Trib3	FW	AAG ACT TGG CTG TGG GAT TCA AGC	190	NM_175093.2
	RV	AGA ACA GGG CCT GAG ATT GTC TGT		
Ddit3/Chop	FW	ACC AAG CAT GAA CAG TGG GCA TCA	164	NM_007837.3
	RV	ATG TAC CGT CTA TGT GCA AGC CGA		
Sqstm1/p62	FW	ACC CTC CAC CAT TGT GAT AGT GCT	109	NM_011018.2
	RV	AAT GCC AAG ACA CTG GGC CTA TCT		
Aqp9	FW	TGA AGG GAC AAG GTA GCC GTT TGA	193	NM_022026.2
	RV	AAA CAG TTG GCA GTG AAG GCA CAC		
Slc7a11	FW	AAA GCA GGT TCC ACA GCG AAG T	227	NM_011990.2
	RV	TGG CCA GCT CCG CAA ATG AAA T		
Cdkn1a	FW	TGC CTG GTT CCT TGC CAC TTC TTA	122	NM_007669.4
	RV	TTC ACT GTC ATC CTA GCT GGC CTT		
Scl40a1	FW	AAC CAG AGT CAC TGT CAT CAG CCA	153	NM_016917.2
	RV	TCG GCC CAA GTC AGT GAA GGT AAA		
Zfp472	FW	TGG GAA AGC CTT CAT CCA ACG TGA	198	NM_153063.3
	RV	AGG TAT GTG GAA CAG GTG AAC GCT		
Nupr1/p8	FW	ACC CAG CAA TGG ATA CAG GAC CTT	149	NM_019738.1
	RV	CTT CTT GCT CCC ATC TTG CCC TTT		
Atf4	FW	TGA GGC TCT GAA AGA GAA GGC AGA	130	NM_009716.2
	RV	AGC ACA AAG CAC CTG ACT ACC CTA		
Dusp1	FW	ATT TGC TGA ACT CGG CAC ATT CGG	154	NM_013642.3
	RV	GGT GGG TGT GTC AAG CAT GAA GTT		
Caspase 4	FW	CCG GAA ACA TGC TTG CTC TTG TCA	118	NM_007609.2
	RV	TCT CGT CAA GGT TGC CCG ATC AAT		
Hmox1	FW	GTG GCC TGA ACT TTG AAA CCA GCA	130	NM_010442.2
	RV	ACA GCA GTC GTG GTC AGT CAA CAT		
Ccl2	FW	ACT GCA TCT GCC CTA AGG TCT TCA	131	NM_011333.3
	RV	TTC ACT GTC ACA CTG GTC ACT CCT		
Gclm	FW	AAA GCA TCC CTG ACA TTG AAG CCC	149	NM_008129.3
	RV	TGT GGG TGT GAG CTG GAG TTA AGA		
IL-1β	FW	TCA CCA TGG AAT CCG TGT CTT CCT	173	NM_008361.3
	RV	ATG TGC CAT GGT TTC TTG TGA CCC		
IFNb1	FW	TGA AGT ACA ACA GCT ACG CCT GGA	163	NM_010510.1
	RV	AGT CCG CCT CTG ATG CTT AAA GGT		

[a]Amplicon length in base pairs.
[b]Genbank accession number of cDNA and corresponding gene, available at http://www.ncbi.nlm.nih.gov
FW, forward primer; RV, reverse primer.

Materials

THC and CBD were obtained from the National Institute on Drug Abuse (Baltimore, MD, USA). Stock solutions of cannabinoids were prepared in ethanol and diluted into culture medium before experiments. Final ethanol concentration in the medium did not exceed 0.1%.

Results

Gene expression profile of BV-2 microglial cells treated with cannabinoids

Samples of mRNA were prepared from BV-2 microglial cells treated for 6 h with CBD or THC (both at 10 μM) or with

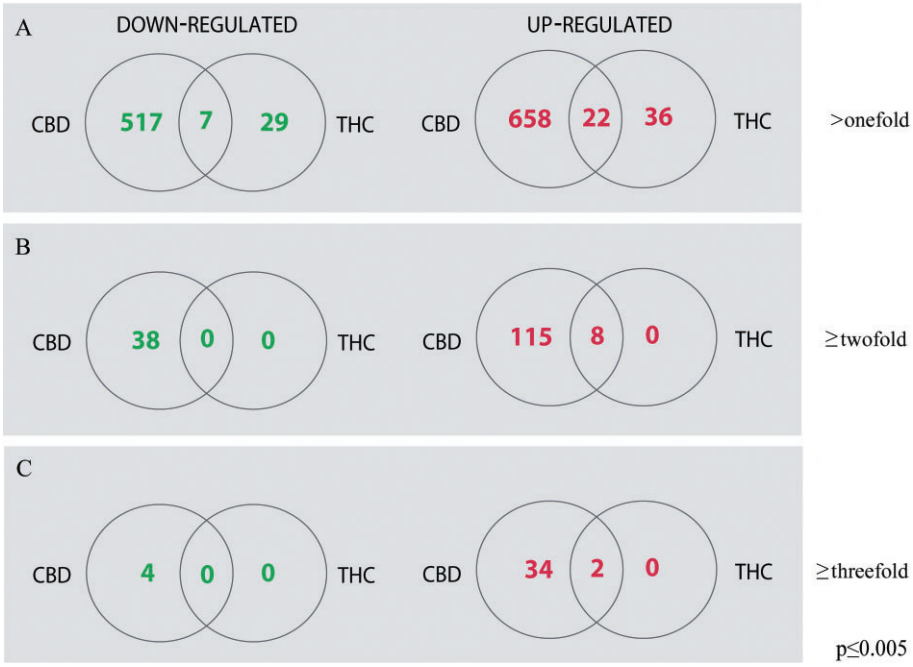

Figure 1

Venn diagrams comparing the number of gene products regulated by CBD and/or THC. The diagrams show the numbers of BV-2 genes that were selectively regulated by 6 h exposure to 10 μM CBD or 10 μM THC. (A) Gene products which were affected by >onefold, (B) by ≥twofold and (C) by ≥threefold. Numbers show the transcripts that were significantly ($P \leq 0.005$) either up- or down-regulated, relative to control untreated cells.

vehicle. The time point of 6 h for gene array analysis was chosen, as it is known to cover the primary gene response (Lund *et al.*, 2006). Moreover, our laboratory used this time point to study the signalling pathways involved in the anti-inflammatory effects of CBD and THC (both at 10 μM), in LPS-treated BV-2 microglial cells (Kozela *et al.*, 2010). Six replicates of each experiment were carried out, resulting in six independent microarrays for each individual treatment or control (18 total arrays). The mRNA profiling was carried out using the MouseRef-8 v1.1 Expression BeadChip Illumina Array, which has >24 000 mouse targets based on the NCBI mouse Reference Sequence Database, including 16 287 constitutive exons/islands based on the splice variants in the mouse transcriptome (MouSDB3) and NCBI LocusLink databases. Of all transcripts, 32% were consistently 'present' across all the 18 arrays. Clustering based on inter-array Pearson correlation coefficient indicated no batch effects between the arrays used in these analyses. Gene classes constitutively expressed included metabolic enzymes, structural proteins, signalling molecules and transcription factors.

Microarray analysis based on a threshold of $P \leq 0.005$, revealed that 1298 transcripts out of the 24 000 targets of the Illumina gene set were differentially regulated across the various treatments. Of these, 680 gene probe sets were up-regulated after 6 h treatment with CBD (Figure 1), whereas a very low number of only 58 gene probe sets were observed to be increased by THC and only 22 of these genes were up-regulated by either CBD or THC. Thus 36 genes were up-regulated by THC and not by the CBD treatment; however, their relative levels did not exceed the twofold induction level in comparison to control. Moreover, CBD had also a much larger effect compared with THC on the number of gene products which were down-regulated: 524 gene products were down-regulated by CBD, 36 by THC and only seven gene products were down-regulated by either THC or CBD ($P \leq 0.005$). In all, 517 probe sets of the chip were down-regulated only by CBD and not affected by THC (Figure 1). When the fold change was set on two, we found that 123 gene products were up-regulated by CBD, and eight of them were up-regulated by either CBD or THC. Thirty-eight gene products were exclusively down-regulated by ≥twofold after CBD treatment while THC did not down-regulate any gene product to this extent.

When the fold change was set on three, we found that 36 genes were up-regulated by CBD and only two of them were up-regulated by THC. Only four genes were exclusively down-regulated to this extent by CBD treatment. THC did not affect any gene to this level. Altogether, both CBD and THC had a greater effect on the number of gene products that were up-regulated than on the number of genes whose expression was repressed (as is best observed for gene products affected by ≥two- or ≥threefold), and the changes in gene expression after THC treatment were much more modest compared to those observed following exposure to CBD. As shown in Figure 1, a relatively small number of genes were selectively responsive to THC (36 up-regulated and 29 down-regulated; >onefold; $P \leq 0.005$) and not to CBD; however, none of these genes reached a level of twofold induction or 50% reduction, in comparison to control.

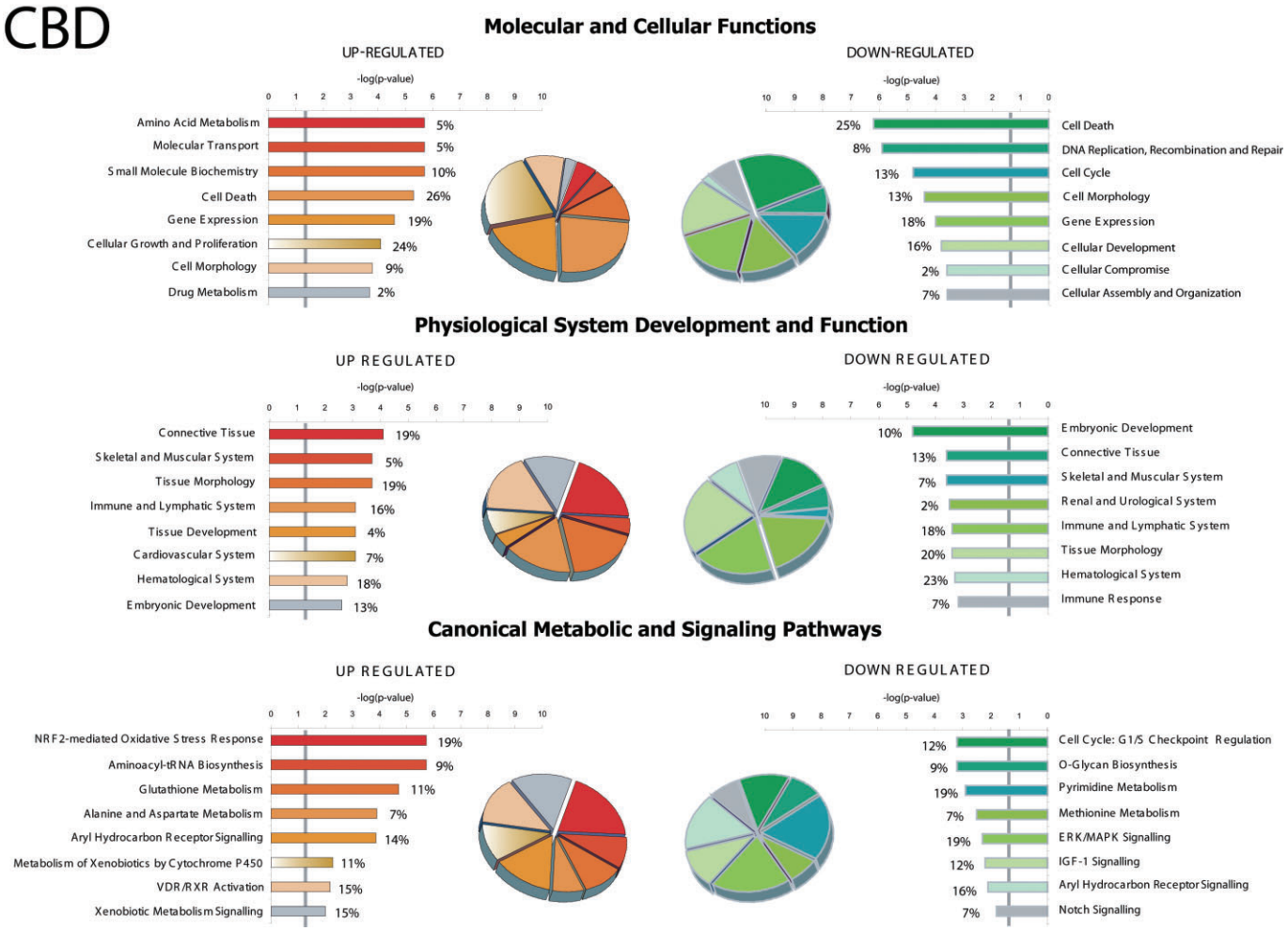

Figure 2

Ingenuity global functional and pathway analysis of the genes affected by CBD treatment. IPA analysis was used to examine the enriched functional classes of up-regulated (red and brown) and down-regulated (green, blue and yellow) genes. The *y* axis shows the top eight most high-level functions and canonical pathways associated with genes regulated in CBD-treated BV-2 microglial cells. The *x* axis displays the mean *P*-value for each associated high-level function and canonical pathway in a –log scale. Increasing value of –log (significance) indicates increased confidence for each category. The vertical gray line in each plot indicates $P < 0.05$. The percentage of genes in each category is shown next to the bars and corresponds to the share in the adjacent pie chart identified by the same colour.

To identify functional subsets of genes and networks regulated by CBD and/or THC, IPA global functional analysis was performed. This detailed analysis revealed that the molecular and cellular functions associated with the CBD-up-regulated genes (≥twofold) included those related to amino acid metabolism, molecular transport, small molecule biochemistry, cell death and gene expression (Figure 2). Furthermore, the CBD-induced canonical metabolic and signalling pathways with the highest significance score include genes involved in Nrf2 (nuclear factor-erythroid 2-related factor 2)-mediated oxidative stress response and aminoacyl-tRNA biosynthesis, as well as genes related to metabolism of GSH, alanine and aspartate. These IPA canonical pathways are in agreement with those we found using the DAVID Bioinformatics Resources and the KEGG database (data not shown). On the other hand, CBD down-regulated the expression of numerous subsets of genes which are known to be associated with diverse biological functions (such as cell death as well as DNA replication, recombination and repair) and canonical metabolic and signalling pathways such as genes related to G1/S checkpoint regulation, metabolism of pyrimidine and methionine as well as ERK/MAPK signalling (Figure 2). As described above, THC affected very few genes; Figure 3 shows that some of the genes down-regulated by THC are related to cellular development, DNA replication, recombination and repair. However, the *P*-value for these effects as well as for other molecular and cellular functions is > 0.05 and did not allow the affected gene pathways to be accurately classified.

Gene-by-gene inspection revealed that genes whose products are known to be active in cellular stress response, regulation of transcription, lipid and amino acid metabolism, as well as membrane transport dominate the CBD-up-regulated transcripts (Table 2). Moreover, CBD treatment induced many transcripts of proteins known to participate in adhe-

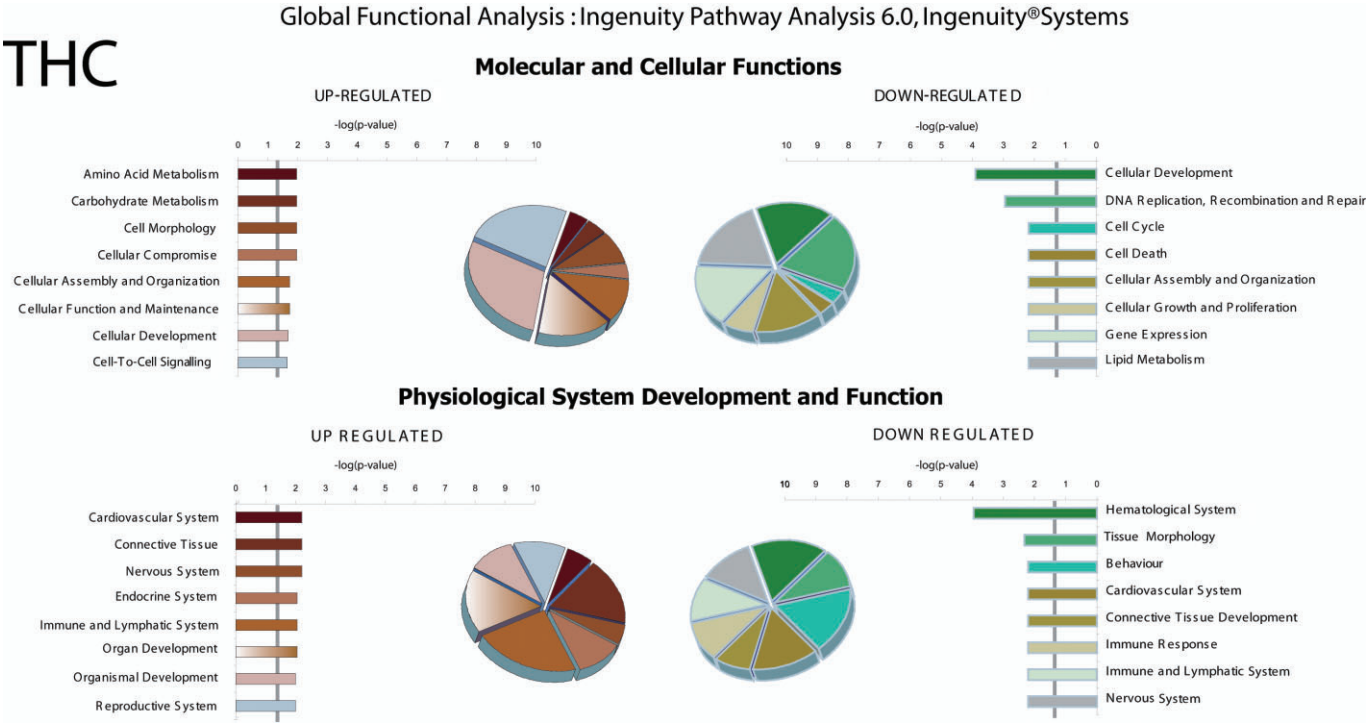

Figure 3

Ingenuity global functional and pathway analysis of the genes affected by THC treatment. IPA analysis was used to examine the enriched functional classes of up-regulated (red and brown) and down-regulated (green, blue and yellow) genes. Details are as indicated in Figure 2.

sion and migration. Genes highly up-regulated by CBD include *tribbles homolog 3* (*Trib3*; 16-fold), *aquaglyceroporin 9* (*Aqp9*; 14-fold), *DNA-damage inducible transcript 3* (*Ddit3*; also known as *Chop/Gadd153*; 12-fold), *cation ion transport regulator 1* (*Chac1*; 10-fold), *growth differentiation factor 15* (*Gdf15*; eightfold), *metallothionein 2* (*Mt2*; eightfold), *sterol O-acyltransferase 2* (*Soat2*; eightfold), *nuclear protein 1* (*Nupr1*; eightfold) and *solute carrier family 7, member 11* (*Slc7a11*; sevenfold). THC increased the expression of some of these genes but to a significantly lower extent. THC-regulated transcripts that were increased by >twofold contained the following six gene transcripts: *Trib3* (3.4-fold), *Chac1* (3.1-fold), *serum amyloid A3* (*Saa3*; 2.7-fold), *lipocalin 2* (*Lcn2*; 2.5-fold), *Gdf15* (2.4-fold) and *homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1* (*Herpud1*; 2.3-fold) as well as two gene transcripts that were up-regulated by twofold, *Aqp9* and *Nupr1*. As described above, the same genes were also up-regulated by CBD. Moreover, except for *Lcn2*, which was affected by both cannabinoids to a similar extent (2.5-fold), all the other genes were affected more strongly by CBD.

The expression of several enzymes which are known to play a key role in cellular defence by increasing the removal of cytotoxic electrophiles and ROS, have been shown to be up-regulated by CBD and less so by THC. These include *Herpud1* (3.3-fold), *Gclm* (threefold), *Gstm6* (2.8-fold), *Hmox*1 (2.7-fold), *Nqo1* (2.4-fold) and *Gstm1* (2.4-fold). Results from our laboratory showed that incubation with 1 μM CBD for 4 h increased ROS formation in BV-2 cells by 215 ± 1% ($P < 0.001$). This assay was performed using the oxidation-sensitive non-fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate, whose oxidized form, 2′,7′-dichlorofluorescein is highly fluorescent, as previously described (Juknat *et al.*, 2005). It is very likely that the up-regulation of these enzymes represents a compensatory response to the ROS formed by CBD.

As for the 38 gene transcripts whose expression was down-regulated by CBD (by 50% or more compared with the control values), they were mainly concentrated within categories of inflammatory chemokines, cell cycle and proliferation, host defence, and regulation of transcription. These transcripts include *zinc finger protein 472* (*Zfp472*; reduced by 80.4%), *chemokine ligand 2* (*Ccl2*; by 72%), *chemokine ligand 7* (*Ccl7*; by 71.4%), *complement component 1, q subcomponent, alpha polypeptide* (*C1qa*; by 70%), *cyclin D1* (*Ccnd1*; by 65.5%) and *cyclin E1* (*Ccne1*; by 63%) (Table 3). As for the transcripts down-regulated by THC, none of them was decreased by 50% or more.

Observing the data presented in Tables 2 and 3, it seems that we can distinguish between two populations of genes: (i) those in which the effects by THC are in the same direction as by CBD (except that the effects with THC are smaller in size) and (ii) those which show CBD selective effects. The latter included genes in all of the groups listed, but especially in the stress response (*Ndrg1*, *Ndrl*, *Sqstm1/p62*, *Ddit4*, *Cathepsin G*, *Nqo1* and *Htatip2*), motility and morphogenesis (*Avil*, *Vwf* and *Pfn2*) and cell cycle and proliferation (*Cdkn1a*, *Cdkn2a*, *Cdkn2b* and *Gadd45a*). This CBD selectivity is even better observed in the list of down-regulated genes where the THC-down-regulated genes never reached the twofold reduction

Table 2

List of genes significantly up-regulated (>twofold) by CBD or THC in BV-2 cells

Gene name	Description	Accession number	Gene array (fold induction) CBD	Gene array (fold induction) THC	qPCR (fold induction)[a] CBD	qPCR (fold induction)[a] THC
Stress response						
Trib3	Tribbles homolog 3 (*Drosophila*)	NM_175093.2	16.3	3.4	15.7 ± 0.3	3.6 ± 0.9
Ddit3/Chop	DNA-damage inducible transcript 3	NM_007837.3	12.0	1.7	12.9 ± 0.5	1.7 ± 0.2
Gdf15	Growth differentiation factor 15	NM_011819.1	8.2	2.4		
Mt2	Metallothionein 2	NM_008630.1	7.9	1.3		
Ndrg1	N-myc downstream regulated gene 1	NM_010884.1	6.5	1.2		
Csprs	Component of Sp100-rs	NM_033616.2	6.5	1.5		
Ndrl	N-myc downstream regulated-like	NM_008681	5.5	1.1		
Cox6a2	Cytochrome c oxidase, subunit VI a, polypeptide 2	NM_009943.2	4.8	1.5		
Herpud1	Homocysteine-inducible, ER stress-inducible, ubiquitin-like domain member 1	NM_022331.1	3.3	2.3		
Sqstm1/p62	Sequestosome 1	NM_011018.1	3.2	1.2	4.8 ± 1.7	1.7 ± 0.1
Ddit4	DNA-damage-inducible transcript 4	NM_029083.1	3.0	1.1		
Ctsg	Cathepsin G	NM_007800.1	2.7	0.9		
Hmox1	Haem oxygenase (decycling) 1	NM_010442.2	2.7	1.4	3.9 ± 1.2	2.1 ± 0.2
Dnmt3l	DNA (cytosine-5-)-methyltransferase 3-like	NM_019448.2	2.7	1.5		
Vegfa	Vascular endothelial growth factor A	NM_009505.2	2.6	1.4		
Hp	Haptoglobin	NM_017370.1	2.6	1.9		
Npn3	Sulphiredoxin 1 homologue (*S. cerevisiae*)	NM_029688.2	2.6	1.3		
Nqo1	NAD(P)H dehydrogenase, quinone 1	NM_008706.1	2.4	1.1		
Htatip2	HIV-1 tat interactive protein 2, homologue (human)	NM_016865.2	2.1	1.0		
Regulation of transcription						
Nupr1/p8	Nuclear protein 1	NM_019738.1	7.9	2.0	10.4 ± 1.5	2.2 ± 0.4
Glrp1	Glutamine repeat protein 1	NM_008132.1	4.3	1.6		
IkB	NF-κB inhibitor β	NM_010908.4	3.5	1.4		
Neurl	Neuralized homolog (*Drosophila*)	NM_021360	3.2	1.0		
NF-kB	Nuclear factor of κ light polypeptide gene enhancer in B-cells 1	NM_008689.2	3.1	1.4		
Jundm2	Jun dimerization protein 2	NM_030887.2	3.0	1.5		
Atf4	Activating transcription factor 4	NM_009716	2.8	1.2	3.5 ± 0.3	1.7 ± 0.1
Chd2	Chromodomain helicase DNA binding protein 2	XM_145698.4	2.8	1.2		
Atf5	Activating transcription factor 5	NM_030693.1	2.5	1.3		
Spic	Spi-C transcription factor (Spi-1/PU.1 related)	NM_011461.2	2.5	1.2		
Relb	Avian reticuloendotheliosis viral (v-rel) oncogene related B	NM_009046.2	2.4	1.3		
Eif4ebp1	Eukaryotic translation initiation factor 4E binding protein 1	NM_007918.2	2.3	1.3		
Atf3	Activating transcription factor 3	NM_007498.2	2.3	1.2		
Hist1h3e	Histone 1, H3e	NM_178205	2.2	1.0		
Hist1h2ac	Histone 1, H2ac	NM_178189.2	2.2	1.1		
Hist1h1c	Histone 1, H1c	NM_015786	2.1	1.2		
Hist2h2be	Histone 2, H2be	NM_178214.1	2.1	1.0		
Mef2b	Myocyte enhancer factor 2B	NM_008578.1	2.1	1.1		
Sox4	SRY-box containing gene 4	NM_009238.2	2.1	0.9		
Hist1h3d	Histone1, H3d	NM_178204.1	2.0	1.0		
Nfe2l1	Nuclear factor, erythroid derived 2,-like 1	NM_008686.3	2.0	1.1		
Metabolic						
Soat2	Sterol O-acyltransferase 2	NM_146064.1	8.0	1.7		
Cox6a2	Cytochrome c oxidase, subunit VI a, polypeptide 2	NM_009943.1	4.8	1.5		

Table 2

Continued

Gene name	Description	Accession number	Gene array (fold induction) CBD	THC	qPCR (fold induction)[a] CBD	THC
Gpt2	Glutamic pyruvate transaminase (alanine aminotransferase) 2	NM_173866.1	4.7	1.3		
Gclm	Glutamate-cysteine ligase, modifier subunit	NM_008129.3	3.0	1.3	2.23 ± 0.06	1.5 ± 0.3
Akr1b7	Aldo-keto reductase family 1, member B7	NM_009731.1	2.9	1.0		
Gstm6	Glutathione S-transferase, μ6	NM_008184.1	2.8	1.4		
Gstm1	Glutathione S-transferase, μ1	NM_010358.5	2.4	1.3		
Adfp	Adipose differentiation related protein	NM_007408.2	2.3	0.9		
Sars1	Seryl-aminoacyl-tRNA synthetase	NM_011319.1	2.2	1.2		
Cars	Cysteinyl-tRNA synthetase	NM_013742.2	2.0	1.2		
Membrane transport and secretion						
Aqp9	Aquaglyceroporin 9	NM_022026.2	14.3	2.0	14.6 ± 3.8	2.5 ± 0.5
Chac1	Cation ion transport regulator 1	NM_026929	10.1	3.1		
Slc7a11	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	NM_011990.1	7.1	1.7	9.2 ± 0.6	1.4 ± 0.2
Slc40a1	Solute carrier family 40 (iron-regulated transporter), member 1	NM_016917.1	6.5	1.7	15.4 ± 0.6	1.6 ± 0.2
Slc6a9	Solute carrier family 6 (neurotransmitter transporter, glycine), member 9	NM_008135.1	6.5	1.6		
Slc1a4	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	NM_018861.2	5.9	1.9		
Slc3a2	Solute carrier family 3 (activators of dibasic and neutral amino acid transport) member2	NM_008577.2	3.8	1.1		
Slc39a4	Solute carrier family 39 (zinc transporter), member 4	NM_028064.2	3.5	1.6		
Stx11	Syntaxin 11	XM_203312.2	3.2	1.4		
Atp2a3	ATPase, Ca++ transporting, ubiquitous	NM_016745.2	2.7	1.2		
Slc2a6	Solute carrier family 2 (facilitated glucose transporter), member 6	NM_172659.1	2.6	1.5		
Slc30a1	Solute carrier family 30 (zinc transporter), member 1	NM_009579.2	2.6	1.0		
Sfxn4	Sideroflexin 4	NM_053198.3	2.1	1.2		
Phosphatases						
Dusp1	Dual specificity phosphatase 1	NM_013642.3	2.8	1.1	3.2 ± 1.5	0.8 ± 0.1
Ptpn14	Protein tyrosine phosphatase, non-receptor type 14	NM_008976.1	2.7	1.1		
Ppap2b	Phosphatidic acid phosphatase type 2B	NM_080555.1	2.2	1.3		
Adhesion and migration						
Angptl6	Angiopoietin-like 6	NM_145154	5.6	1.2		
Saa3	Serum amyloid A 3	NM_011315	4.4	2.7		
Col4a1	Procollagen, type IV, α1	NM_009931.1	3.5	1.0		
Sned1	Sushi, nidogen and EGF-like domains 1	NM_172463.3	2.8	1.2		
Clecsf9	C-type (calcium dependent, carbohydrate recognition domain) lectin	NM_019948.1	2.3	1.7		
Mmp23	Matrix metalloproteinase 23	NM_011985.1	2.5	1.2		
Pcdha1	Protocadherin α 1	NM_054072	2.4	1.1		
Pcdha11	Protocadherin α 11	NM_009960	2.2	1.1		
Motility and morphogenesis						
Avil	Advillin	NM_009635.2	2.6	1.0		
Vwf	Von Willebrand factor homolog ue	NM_011708.2	2.6	1.1		
Pfn2	Profilin 2	NM_019410.2	2.3	1.1		
Apoptosis						
Tnfaip2	Tumour necrosis factor α-induced protein 2	NM_009396.1	3.3	1.8		
Lcn2	Lipocalin 2	NM_008491.1	2.5	2.5		

Table 2

Continued

Gene name	Description	Accession number	Gene array (fold induction) CBD	Gene array (fold induction) THC	qPCR (fold induction)[a] CBD	qPCR (fold induction)[a] THC
Traf1	TNF receptor-associated factor 1	NM_009421.2	2.3	1.2		
Casp4	Caspase 4, apoptosis-related cysteine protease	NM_007609.1	2.1	1.2	3.2 ± 0.6	1.1 ± 0.3
Pdcd1lg1	Programmed cell death 1 ligand 1	NM_021893.2	2.1	1.5		
Cell cycle and proliferation						
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	NM_007669.4	3.7	0.7	3.24 ± 0.6	1.0 ± 0.1
Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	NM_007670.2	2.9	1.1		
Gadd45a	Growth arrest and DNA-damage-inducible 45 α	NM_007836.1	2.3	1.2		
Cdkn2a	Cyclin-dependent kinase inhibitor 2A	NM_009877.1	2.1	0.7		
G protein-coupled receptors						
Ptgir	Prostaglandin I receptor (IP)	NM_008967.1	5.4	1.3		
Kinases						
Plk2	Polo-like kinase 2	NM_152804.1	2.2	1.1		
Regulation of translation						
Myd116	Myeloid differentiation primary response gene 116	NM_008654.1	2.7	1.1		
Cpeb1	Cytoplasmic polyadenylation element binding protein 1	NM_007755.1	2.2	1.2		

[a]Genes were validated by qPCR, using β2-microglobulin mRNA as a reference gene.

(50% decrease). The gene products *Chst 10* (reduced by 44%), *Ly86* (by 41%) and *Pscdbp* (by 41%) were the main THC-down-regulated genes.

Validation of microarray results

Several genes that were identified by microarray analysis as differentially regulated were subjected to validation by qPCR using *β2-microglobulin* (*B2m*) as a reference gene. Individual sets of genes were selected to be validated according to their association with the amino acid deprivation pathway and cyst(e)ine uptake as well as with the oxidative stress response. The qPCR assays were repeated four times using at least three mRNA preparations from independent experiments. The results are expressed as fold change relative to control levels. We found that in almost all cases, there is a very good agreement between the microarray and the qPCR results in terms of direction of change as well as of its magnitude. For example, the qPCR data (Table 2) show that CBD up-regulated the expression of *Trib3*, *Slc40a1*, *Aqp9*, *Ddit3/Chop/Gadd153*, *Nupr1/p8*, *Slc7a11*, *Sqstm1*, *Atf4*, *Hmox1*, *Cdkn1a*, *Dusp1* and *Casp4*. The qPCR data show that THC treatment up-regulated the mRNA expression of *Trib3*, *Aqp9*, *Nupr1* and *Hmox1*. qPCR results also show that CBD down-regulated the expression of *Zfp472* and *Ccl2* . All these results are in complete agreement with the gene array data. The latter result (regarding *Ccl2*) differs from the data reported by Kozela *et al*. (2010) due to a change to a better set of primers oriented toward a different part of the sequence using a newer gene accession number.

Numerous molecular targets (some overlapping and some are not) have been reported for CBD and for THC (De Petrocellis and Di Marzo, 2010; De Petrocellis *et al*., 2011), including, but not limited to, CB_1 and CB_2 receptors, GPR55, GPR18, the transient receptor potential of vanilloid type-1 and type-2 (TRPV1; TRPV2) channels, the peroxisome proliferator-activating receptors (PPARs) and fatty acid amide hydrolase (FAAH). We and others found that unstimulated BV-2 microglial cells express CB_1 and CB_2 receptors, GPR55, GPR18 and TRPV2 channels (Pietr *et al*., 2009; McHugh *et al*., 2010; Stella, 2010; Rimmerman *et al*., 2012). In contrast to the relatively low abundance of CB_1 mRNA, we found that both CB_2 and *GPR55* mRNAs are present at relatively high levels in BV-2 cells as well as in unstimulated primary microglia in culture (Pietr *et al*., 2009). Using the gene array analysis in this study, we have observed that the relative levels of CB_1, CB_2, *GPR18*, *TRPV2* and *FAAH* did not exceed the twofold induction or 50% reduction by the cannabinoid treatment and were not affected to a significant extent. Only the effect of CBD on *PPARγ2* (+40%) was found to be statistically significant ($P < 0.01$). qPCR studies strengthened this observation showing that the changes observed in most of these gene products (CB_1, CB_2, *GPR55* and *GPR18*) were small and did not reach a statistical significance.

In a recent paper from our group, Kozela *et al*. (2010) described the effects of CBD and THC on LPS-activated BV-2 cells, showing differential anti-inflammatory activities and mechanisms for these cannabinoids. The signalling pathways involved in these activities are composed of different factors and regulators, like IL-1β, IL-6, IRAK1, IFN-β, suppressors of cytokine signalling 3 (SOCS3) as well as signal transducers and activators of transcription 1 and 3 (STAT1 and STAT3). Using gene array analysis, we found that CBD and THC did not statistically significantly affect the expression of these genes in surveillant (resting) cells; their relative levels did not

Table 3

List of genes from gene arrays, down-regulated by CBD (by more than 50%)

Gene name	Description	Accession number	Fold change and percentage of reduction CBD	THC
Inflammatory chemokines and receptors				
Ccl2	CCL2	NM_011333.1	−3.6 (72%)	−1.0 (0%)
Ccl7	CCL7	NM_013654	−3.5 (71.4%)	1.2
Cxcl14	CXCL14	NM_019568	−2.6 (61.5%)	−1.4 (29%)
Cx3cr1	CX3CR1	NM_009987.2	−2.1 (52.4%)	−1.3 (23%)
Ccl6	CCL6	NM_009139.1	−2.1 (52.4%)	−1.0 (0%)
Ccl9	CCL 9	NM_011338	−2.0 (50%)	−1.2 (17%)
Membrane transport and secretion				
Kctd12	Potassium channel tetramerization domain containing 12	NM_177715.2	−2.5 (60%)	−1.0 (0%)
Slc25a22	Solute carrier family 25 (mitochondrial carrier: glutamate), member 22	NM_026646.1	−2.1 (52.4%)	−1.0 (0%)
Slc39a10	Solute carrier family 39 (zinc transporter), member 10	NM_172653.2	−2.1 (52.4%)	−1.2 (17%)
Slc5a6	Solute carrier family 5 (sodium-dependent vitamin transporter), member 6	NM_177870.2	−2.1 (52.4%)	−1.1 (9%)
Metabolic				
Usp2	Ubiquitin specific protease 2	NM_198091.1	−2.3 (57%)	−1.3 (23%)
Siat7d	Sialyltransferase 7	NM_011373.1	−2.2 (55%)	−1.2 (17%)
Chst10	Carbohydrate sulphotransferase 10	NM_145142.1	−2.2 (55%)	−1.8 (44%)
Mat2a	Methionine adenosyltransferase II, $\alpha 2$	NM_145569	−2.0 (50%)	−1.3 (23%)
Cell cycle and proliferation				
Ccnd1	Cyclin D1	NM_007631.1	−2.9 (65.5%)	−1.2 (17%)
Ccne1	Cyclin E1	NM_007633.1	−2.7 (63%)	−1.2 (17%)
Idb1	Inhibitor of DNA binding 1	NM_010495.1	−2.2 (54.5%)	−1.5 (33%)
Cdc6	Cell division cycle 6 homologue (*S. cerevisiae*)	NM_011799.1	−2.1 (52.4%)	−1.0 (0%)
Ccne2	Cyclin E2	NM_009830.1	−2.0 (50%)	−1.2 (17%)
Cdc25a	Cell division cycle 25 homologue A (*S. cerevisiae*)	NM_007658	−2.0 (50%)	−1.0 (0%)
Host defence and adaptive response				
C1qa	Complement component 1, q subcomponent, α polypeptide	NM_007572	−3.3 (70%)	−1.3 (23%)
Ebi2	EPSTEIN-Barr virus induced gene 2	NM_183031.1	−2.4 (58%)	−1.3 (23%)
Igfbp4	Insulin-like growth factor binding protein 4	NM_010517.2	−2.4 (58%)	−1.3 (23%)
Tlr13	Toll-like receptor 13	NM_205820.1	−2.3 (56.5%)	−1.3 (23%)
Ly86	Lymphocyte antigen 86 (Ly86), mRNA.	NM_010745.1	−2.0 (50%)	−1.7 (41%)
Dok2	Docking protein 2 (Dok2), mRNA.	NM_010071.1	−2.0 (50%)	−1.1 (9%)
Regulation of transcription				
Zfp472	Zinc finger protein 472	NM_153063.3	−5.1 (80.4%)	1.2
Fos	FBJ osteosarcoma oncogene	NM_010234.2	−2.5 (60%)	−1.2 (17%)
Mef2c	Myocyte enhancer factor 2C	NM_025282.3	−2.1 (52.4)	−1.2 (17%)
Tle1	Transducin-like enhancer of split 1, homologue of *Drosophila* E	NM_011599.2	−2.0 (50%)	−1.3 (23%)
Ase1	CD3E antigen, ε polypeptide associated protein	NM_145822.1	−2.0 (50%)	−1.2 (17%)
Regulation of translation				
Eif5a	Eukaryotic translation initiation factor 5A	NM_181582.4	−2.6 (61.5%)	−1.2 (17%)
Adhesion and migration				
Pscdbp	Pleckstrin homology, Sec7 and coiled-coil domains, binding protein	NM_139200.2	−2.1 (52.4%)	−1.7 (41%)
Motility and morphogenesis				
Rab3il1	RAB3A interacting protein (rabin3)-like 1	NM_144538.1	−2.1 (52.4%)	−1.3 (23%)
Rin2	Ras and Rab interactor 2	NM_028724.2	−2.0 (50%)	−1.1 (9%)
Unidentified				
4921501M20Rik	RIKEN cDNA 4921501M20 gene (4921501M20Rik), mRNA.	NM_028728.1	−2.7 (63%)	−1.2 (17%)
0910001A06Rik	RIKEN cDNA 0910001A06 gene (0910001A06Rik), mRNA.	NM_144846.2	−2.1 (52.4%)	−1.1 (9%)
C85492	expressed sequence C85492 (C85492), mRNA.	NM_153540.2	−2.1 (52.4%)	−1.0 (0%)

List of genes down-regulated at least twofold by CBD in resting BV-2 microglial cells. Fold decrease for each gene was calculated according to the formula: Fold change = $(-1) \times 2^{-(\text{signal log ratio})}$

exceed the twofold induction or 50% reduction. Analysing the expression of these genes by qPCR, we found that indeed in most cases, they were not significantly affected by either CBD or THC. On the other hand, the qPCR data showed that *IL-1β* was highly down-regulated by CBD and THC (80 ± 3% and 74 ± 10% respectively; $n = 5$; $P < 0.001$) and *IFNb1* was up-regulated by CBD (120 ± 30%; $n = 3$; $P < 0.005$) and down-regulated by THC by 65 ± 8% ($n = 3$; $P < 0.05$).

Discussion

Cannabinoids have antioxidant, neuroprotective, proapoptotic and anti-tumour properties (Klein *et al.*, 1998; Galve-Roperh *et al.*, 2000; McKallip *et al.*, 2002; Cabral and Staab, 2005; van der Stelt and Di Marzo, 2005; Klein and Cabral, 2006; Massi *et al.*, 2006; Kozela *et al.*, 2010; Rieder *et al.*, 2010). Several cannabinoids were effective in treating pain, glaucoma, wasting, nausea and vomiting as well as AIDS, and spasticity in multiple sclerosis (Pertwee, 2002; Guzman, 2003; Di Marzo and De Petrocellis, 2006; Kogan and Mechoulam, 2007). Various cannabinoids also have immunomodulatory and anti-inflammatory effects which are probably mediated via their activity on various types of immune cells (such as B cells, T cells, macrophages, NK cells, dendritic cells) and microglia (Klein *et al.*, 1998; 2000; Croxford and Yamamura, 2005; Kozela *et al.*, 2010; Rieder *et al.*, 2010). These cannabinoids exert their immunosuppressive activity by induction of apoptosis, inhibition of cell proliferation, down-regulation of cytokines and chemokine production and release, and induction of regulatory T cells (Croxford and Yamamura, 2005; Klein and Cabral, 2006; Kozela *et al.*, 2010; Rieder *et al.*, 2010). Moreover, both THC and CBD have anti-inflammatory activities, even though CBD does not seem to be active on either CB_1 or CB_2 receptors, suggesting a CB_1/CB_2 receptor-independent mechanism and the involvement of other cell targets (Kozela *et al.*, 2010; O'Sullivan and Kendall, 2010).

However, none of these studies addressed the effects of cannabinoids and especially of the non-psychotropic CBD, at the level of genome-wide expression. The present study shows the effect of treatment with the two major cannabinoids present in *Cannabis*, CBD and THC, on genome-wide mRNA levels in resting murine BV-2 microglial cells. We identified 1204 differentially expressed genes in response to CBD treatment, which were associated with many different functions, including stress, inflammatory response, membrane transport, adhesion and migration, cell cycle and proliferation. Moreover, we show that CBD affects the expression of many more gene transcripts (by about 18-fold) in comparison to THC and that the changes in the level of transcription are also much higher with CBD. Only 36 genes were up-regulated and 29 down-regulated exclusively by THC and not by CBD. Moreover, the effects on the expression of these genes were small, compared with the effects of CBD.

We show here, that CBD, and less so THC, repressed the expression of an important subset of pro-inflammatory genes, especially the chemokine ligands, CCL2, CCL7, CXCL14, CCL6, CCL9 and the chemokine receptor CX3CR1. These effects are in agreement with reports showing that cannabinoids can modulate the functional activities of immune cells and exert immunosuppressive effects mainly by inhibition of cytokine and chemokine production, induction of apoptosis and inhibition of cell proliferation (Klein and Cabral, 2006; Kozela *et al.*, 2010; Rieder *et al.*, 2010). Among the chemokines found to be down-regulated, the chemokine-receptor network, involving the chemokine CCL2(MCP-1/JE) and its receptor CCR2, is by far the best known for its involvement in (i) neuroprotection (such as reduction in CCL2-mediated immune cell infiltration after stroke or traumatic brain injury); (ii) neurotransmission (such as increase of cell excitability, dopamine release and locomotor activity in CCL2-injected rats); and (iii) neurogenesis (by CCL2 induction of stem-cell migration into sites of damage in the injured brain, and directing differentiation of precursor cells into neural cells) (Chintawar *et al.*, 2009; Magge *et al.*, 2009; Semple *et al.*, 2010). The CCL2/CCR2 signalling is involved not only in these physiological processes but also in regulating immune cell infiltration into injured areas of the CNS (Semple *et al.*, 2010). Moreover, the observation that CBD treatment down-regulates the expression of CCL2 mRNA is in agreement with our previous results showing that CBD decreased by 58% the LPS-up-regulated CCL2 mRNA expression in BV-2 cells (Kozela *et al.*, 2010).

The list of CBD-up-regulated genes and the IPA analysis reveal that the CBD-specific gene expression profile is showing changes associated with oxidative stress and GSH depletion which normally occur under nutrient limiting conditions or P450-dependent biotransformation of xenobiotics. Furthermore, these CBD-up-regulated genes are known to be controlled by nuclear factors usually involved in the regulation of stress response and cell death, mainly Nrf2 and the activating transcription factor 4 (ATF4). These observations indicate that CBD, but less so THC, induces a cellular stress response and that this response seems to underlie its immunosuppressive activity. Indeed, the gene expression profile induced by treatment with CBD on resting BV-2 microglial cells, shows changes in several families of genes known to be regulated/activated by ATF4, including those encoding amino acid biosynthetic enzymes, amino acid transporters and aminoacyl-tRNA synthetases. Of the subset of genes that were significantly up-regulated in response to CBD, five genes (*Trib3, Ddit3/Chop/Gadd153, Atf4, Atf3* and *Slc7a11*) are known to contain amino acid response elements (AAREs), and to respond to amino acid deprivation, suggesting an effect of CBD on the GCN2/eIF2α/ATF4 pathway (Wek *et al.*, 2006; Kilberg *et al.*, 2009). The amino acid deprivation pathway is initiated by the amino acid sensor GCN2 [general control non-derepressible 2; also known as eukaryotic initiation factor-2α (eIF2α) kinase 4], which leads to phosphorylation and inactivation of eIF2α, suppressing global mRNA translation but allowing translation of ATF4 with the subsequent induction of genes for amino acid biosynthesis and transport (Taylor, 2009). Moreover, *Gclm* (*glutamate-cysteine ligase, modifier subunit*), *Slc1a4* [*solute carrier family 1 (glutamate/neutral amino acid transporter), member 4*], *Slc3a2* [*solute carrier family 3 (activator of dibasic and neutral amino acid transporter), member 2*] and *Cars* (*cysteinyl-tRNA synthetase*) are additional related genes found to be up-regulated by CBD. These genes are involved in cyst(e)ine transport and its metabolism. Up-regulation of these genes (including *Slc7a11*) would facilitate cysteine uptake by the cells and lead to increased GSH synthesis as part of the normal response of

the cell to amino acid deficiency or to other stress stimuli (such as proteasome inhibition) that activates GCN2/eIF2α/ATF4 pathway. Other amino acid-related genes found to be up-regulated by CBD are *Slc6a9* [*solute carrier family 6 (neurotransmitter transporter, glycine), member 9*], *Sars* (*seryl-aminoacyl-tRNA synthetase*), *Chac1* and *Gadd45a* (*growth arrest and DNA-damage-inducible 45α*). The latter gene product is known to encode a protein that inhibits proliferation and suppresses growth, functions that serve as protecting events during amino acid deficiency. Our present results are, thus, in agreement with reports that show up-regulation of genes of the amino acid response pathway leading to eIF2α phosphorylation and increased ATF4 translation, in response to cysteine deprivation or amino acid-deficient media in HepG2 human hepatocarcinoma cells (Lee *et al.*, 2008c; Palii *et al.*, 2009).

The GCN2/eIF2α/ATF4 pathway has been also found to be activated by proteosome inhibition (Wek *et al.*, 2006). In this regard, we found that CBD up-regulates *Sqstm1/p62* expression (*sequestosome 1*; 3.2-fold), a multifunctional ubiquitin-binding protein, which has been implicated in a variety of processes including cell signalling, receptor internalization, protein turnover (Seibenhener *et al.*, 2007) and autophagic clearance of ubiquitinated protein aggregates (Pankiv *et al.*, 2007; Ding and Yin, 2008). Recent reports showed that Sqstm1/p62 can be employed as an autophagic marker as there is a correlation between inhibition of autophagy and increased levels of Sqstm1/p62 (Pankiv *et al.*, 2007).

Another gene up-regulated by CBD is *Nupr1/p8* (eight-fold). *Nupr1* was found to be induced by ATF4 in response to various cellular stressors. Nupr1 expression has been reported to be associated with enhanced transcriptional activation of genes downstream of ATF4 (like CHOP and TRIB3), suggesting that Nupr1/p8 promotes the transcription of stress-regulated genes via positive feedback on the ATF4 pathway (Jin *et al.*, 2009). According to these results, CBD and to a lesser extent THC, activates the GCN2/eIF2α/p8/ATF4/CHOP-TRIB3 pathway, known to lead to autophagy as well as to apoptotic cell death. It is important to recall that the group of Velasco reported induction by THC of cell death associated with stress-related gene expression via the p8-ATF4-TRIB3 pathway, in U87MG human astrocytoma and C6 glioma cells (Carracedo *et al.*, 2006a,b; Salazar *et al.*, 2009).

As mentioned above, the CBD-induced pathways include genes involved in Nrf2-mediated oxidative stress. Nrf2 is a redox-sensitive transcription factor that plays a central role in cellular defence against oxidative stress- and cytotoxic electrophile-induced insults through transcriptional activation of an array of genes, including phase II detoxifying enzymes, antioxidants and transporters. Nrf2 binds to the antioxidant response element/electrophile response element (ARE/EpRE), located in the promoter region of genes encoding phase II detoxifying or antioxidant enzymes and related stress-response proteins (Kobayashi and Yamamoto, 2006). These include among others, haem oxygenase-1 (Hmox1), GSH S-transferase (GST), GSH peroxidase, NAD(P)H:quinone oxidoreductase (Nqo1) and glutamate-cysteine ligase (Gcl), which play key roles in cellular defence by enhancing the removal of cytotoxic electrophiles and ROS, and by maintaining GSH homeostasis. From the list of up-regulated genes (Table 2) *Herpud1*, *Hmox1*, *Nqo1* and *Gclm* are genes known to be regulated in response to oxidative stress via the (ARE/EpRE)/Nrf2 system. One of the mechanisms of defence against the toxicity of ROS is the GST-catalysed conjugation of xenobiotics with endogenous GSH (Shih *et al.*, 2003). Indeed, we found up-regulation by CBD of *GST mu6* and *GST mu1* mRNA expression by 2.8- and 2.4-fold respectively.

Nrf2 also regulates the expression of two cellular transporters, the cysteine glutamate exchange transporter (that buffers cysteine influx against GSH efflux) and the Mrp1 transporter (functions to exclude the entry of xenobiotic metabolites) (Cullinan and Diehl, 2006). Nrf2 belongs to a subclass of transcription factors that are incapable of self-dimerization. Thus, sequence-specific DNA binding and subsequent induction of target gene transcription requires association of Nrf2 with other transcription factors like ATF4 that has been identified as an Nrf2 interacting protein (He *et al.*, 2001). Indeed, Nrf2 is a potential activator at the ATF4 promoter (Cullinan and Diehl, 2006).

Among the mediators up-regulated by Nrf2, the antioxidant enzyme Hmox1 (whose gene is up-regulated by CBD) has been shown to modulate inflammation and innate immunity (see Kim *et al.*, 2010). Interestingly, Nrf2-dependent Hmox1 was found to inhibit CCL2 (shown to be down-regulated by CBD) suggesting that activation of Nrf2 negatively regulates the expression of pro-inflammatory chemokines, via Hmox1 up-regulation. This up-regulation of *Hmox1* and down-regulation of *Ccl2* mRNAs suggest the possible involvement of CBD in the regulation of the Nrf2/Hmox1 axis, a pathway relevant for the restoration of the redox homeostasis and for the modulation of inflammatory responses (Innamorato *et al.*, 2008, 2009; Singh *et al.*, 2010). Tzima *et al.* (2009) reported that myeloid Hmox1 regulates IFN-β production establishing Hmox1 as a critical early mediator of the innate immune response in mice. Moreover, aside from our findings on the up-regulation of *Hmox1* by CBD, we found (by qPCR) that CBD enhanced the expression of *IFNb1* mRNA. This is a very significant result, due to the fact that IFN-β is currently used in the treatment of multiple sclerosis, a chronic inflammatory disease of the CNS (Bates, 2011; Rudick and Goelz, 2011) and was shown to diminish symptoms of experimental autoimmune encephalomyelitis (EAE), an multiple sclerosis model in mice (Galligan *et al.*, 2010). These results could explain the effect of CBD in ameliorating the EAE disease symptoms as previously reported (Kozela *et al.*, 2011).

As reported by Kozela *et al.* (2010), STAT molecules have a role in CBD-induced anti-inflammatory effects. In this regard, it was reported that the down-regulation of the expression of *Ccl2* mRNA by CBD (but not THC) in LPS-treated cells is in agreement with the increase in STAT3 activation and with the decrease in STAT1 phosphorylation after CBD treatment. Accordingly, our current results show down-regulation of *Ccl2* mRNA by CBD and not by THC. As CBD down-regulates the LPS-up-regulated SOCS3 expression, the inducible effect of CBD on the activation of STAT3 could be mediated via the CBD effect on SOCS3. These effects of CBD on STAT3 and on SOCS3 could underlie the potential benefits of CBD in the treatment of neuroinflammatory diseases.

We found that CBD increases ROS formation in BV-2 cells. This result is in agreement with our gene array analysis, where we found up-regulation of the expression of *Herpud1*,

Gclm, *Gstm6*, *Hmox*, *Nqo1* and *Gstm1* by CBD. These are key enzymes which are involved in cellular defence by increasing the removal of cytotoxic electrophiles and ROS, leading to GSH homeostasis. These results are in agreement with previous reports showing the ability of CBD to induce ROS production as well as enhancing apoptosis via an oxidative stress-dependent mechanism (Massi *et al.*, 2006; Wu *et al.*, 2008; Lee *et al.*, 2008a). In our previous work, we reported that THC (0.1–1.0 μM) is able to enhance cellular damage from oxidative stress in C6 glioma cells, when incubated in the presence of the synthetic cell permeable quinone Q_c^b (2-phenyl-4-[butylamino]-naphtholquinoline-7,12-dione), a reagent that generates ROS. No effect of THC on release of LDH was observed in the absence of this ROS generator, demonstrating that THC by itself is a weak generator of ROS. Moreover, THC was found to be a radical scavenger and is likely to serve as a trap for ROS (Goncharov *et al.*, 2005).

Lee *et al.* (2008b) reported that induction of ROS by cocaine treatment of AF5, a neural progenitor cell line, contributes to eIF2α-ATF4-mediated cyclin A2 down-regulation, leading to cocaine-induced inhibition of proliferation. We have found that CBD, and less so THC, down-regulates the expression of *cyclins D1* and *E1* in resting BV-2 microglial cells. Moreover, CBD induces the expression of *cyclin-dependent kinase inhibitor 1A* (*Cdkn1a*, also called *p21*; Table 2), a gene product that regulates many cellular processes such as cell cycle arrest, DNA replication and repair, cell differentiation, senescence and apoptosis. Interestingly, Gadd45a, a gene product that binds directly to Cdkn1a/p21, is also up-regulated by CBD. In this regard, Cdkn1a/p21 has been shown to inhibit apoptosis signal-regulating kinase-1 (ASK-1)-mediated activation of JNK and to induce resistance to cell death under conditions of oxidative stress both *in vivo* and *in vitro* (Langley *et al.*, 2008). Recent evidence suggests that p21-dependent cell survival under oxidative stress is mediated through the activation of the Nrf2 signalling pathway, showing that Nrf2 (and its downstream genes) is up-regulated by p21 via a direct interaction between these two proteins (Chen *et al.*, 2009). The mRNA and protein expression of p21 was also reported to be up-regulated in response to amino acid deprivation in HepG2 human hepatoma cells (Leung-Pineda *et al.*, 2004).

Regulated water transport is important for mitochondrial homeostasis. It is known that changes in mitochondrial volume are associated with normal physiological processes as well as with pathological conditions such as those involved in ROS formation and cell injury. Aquaporin water channels are responsible for water transport (import and export) through mitochondrial and cell membranes (see Gena *et al.*, 2009). In this regard, we found that CBD up-regulated genes related to water, iron and zinc transport, including *Aqp9* (14-fold), *Slc40a1* (*iron-regulated transporter*; 6.5-fold) and the zinc transporters *Slc39a4* and *Slc30a1* (3.5-fold and 2.6-fold respectively) (Table 2). In addition to water, *Aqp9* contributes to the transport of small solutes (such as glycerol, lactate and urea), and participates in osmotic swelling induced by apoptotic stimuli (Lee and Thevenod, 2006). It is interesting to note that, following metabolic stress, Aqp9 expression is induced in pyramidal neurons, which do not express this channel under normal physiological conditions (see Badaut, 2010).

In summary, in the present study we show that CBD affected the expression of many more genes, than those affected by THC. We found that CBD induced a robust response related to oxidative stress and GSH deprivation, which seems to be controlled by Nrf2 and ATF4 transcription factors. Concerning the mechanism underlying the CBD actions, it seems that CBD treatment leads to depletion of intracellular GSH, activating the GCN2/eIF2α/p8/ATF4/CHOP-TRIB3 pathway, accompanied by generation of ROS via the (EpRE/ARE)-Nrf2/ATF4 system, and to regulation of the Nrf2/Hmox1 axis, involved in modulation of redox homeostasis and inflammatory responses. The anti-inflammatory effects of CBD seem to correlate with up-regulations of the expression of *Hmox1* and *IFNb1*, and down-regulation of the expression of *Ccl2*, via the IFN-β-STAT pathway.

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Conflicts of interest

The authors state no conflict of interest.

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