Neurochemistry International 61 (2012) 923–930

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Cannabidiol affects the expression of genes involved in zinc homeostasis in BV-2 microglial cells

Ana Juknat [a], Neta Rimmerman [a,b], Rivka Levy [b], Zvi Vogel [a,b,*], Ewa Kozela [a,b]

[a] 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
[b] Neurobiology Department, Weizmann Institute of Science, Rehovot 76100, Israel

ARTICLE INFO

Article history:
Available online 9 December 2011

Dedicated to the memory of Dr. Marshall W. Nirenberg.

Keywords:
Cannabidiol
Metallothionein
Δ^9-Tetrahydrocannabinol
Zinc transporters
Matrix metalloproteinase 23

ABSTRACT

Cannabidiol (CBD) has been shown to exhibit anti-inflammatory, antioxidant and neuroprotective properties. Unlike Δ^9-tetrahydrocannabinol (THC), CBD is devoid of psychotropic effects and has very low affinity for both cannabinoid receptors, CB_1 and CB_2. We have previously reported that CBD and THC have different effects on anti-inflammatory pathways in lipopolysaccharide-stimulated BV-2 microglial cells, in a CB_1/CB_2 independent manner. Moreover, CBD treatment of BV-2 cells, was found to induce a robust change in the expression of genes related to oxidative stress, glutathione deprivation and inflammation. Many of these genes were shown to be controlled by Nrf2 and ATF4 transcription factors.

Using the Illumina MouseRef-8 BeadChip platform, DAVID Bioinformatics and Ingenuity Pathway Analysis, we identified functional sets of genes and networks affected by CBD. A subset of genes was found to be regulated by the metal responsive element (MRE)-binding transcription factor-1 (MTF-1) and is shown to be related to zinc homeostasis. We found that CBD upregulates the expression of the mRNAs for *metallothionein 2* (*Mt2*), *N-myc-downstream regulated gene 1* and *matrix metalloproteinase 23* as well as of the zinc transporters *ZnT1/Slc30a1* and *Zip4/Slc39a4* but downregulates the expression of the mRNA for the zinc transporter *Zip10/Slc39a10* as well as for the *zinc finger protein 472*. Among these genes, ZnT1, Mt2 and the zinc transporters ZIPs are known to function together to control the intracellular zinc concentration.

These results show that CBD, but much less so THC, affects the expression of genes involved in zinc homeostasis and suggest that the regulation of zinc levels could have an important role through which CBD may exert its antioxidant and anti-inflammatory effects.

1. Introduction

1.1. Cannabinoids and immune cells

Preparations derived from *Cannabis sativa* (marijuana and hashish) have been used for centuries as recreational drugs as well as medicinal agents, due to their psychotropic and therapeutic properties (for reviews, see Earleywine (2002), Kogan and Mechoulam (2007), Murray et al. (2007) and Pertwee (2009)). To date, over 60 phytocannabinoids have been identified. The two most abundant phytocannabinoids in *Cannabis* preparations are Δ^9-tetrahydrocannabinol (THC), the major psychoactive constituent, and cannabidiol (CBD), which is not psychoactive.

Many cannabinoids were shown to posses immunosuppressive and anti-inflammatory properties and to modulate various activities of immune cells (Klein et al., 1998; McKallip et al., 2002; Cabral and Staab, 2005; Croxford and Yamamura, 2005; Klein and Cabral, 2006; Kozela et al., 2010, 2011; Rieder et al., 2010; Juknat et al., 2011). In addition, a large number of reports showed that several cannabinoids have proapoptotic, neuroprotective and anti-tumor properties (Galve-Roperh et al., 2000; van der Stelt and Di Marzo, 2005; Massi et al., 2006).

To date, two cannabinoid receptors have been characterized, the CB_1 and the CB_2 receptors. The CB_1 receptor is mostly localized in neural cells and mediates the psychoactive effects of THC, while the CB_2 receptor is highly expressed in immune cells and is involved in immunomodulation (for reviews, see Cabral et al. (2008), Cabral and Griffin-Thomas (2009) and Stella (2010)). THC is equally efficient at both of these receptors (Rhee et al., 1997) and has been reported to have effects on both the nervous and the immune systems (Cabral and Staab, 2005; Le Foll and Goldberg, 2005; Cabral et al., 2008; Woelkart et al., 2008; Cabral and Griffin-Thomas, 2009). CBD, unlike THC, has low

Abbreviations: CBD, cannabidiol; Mt, metallothionein; THC, Δ^9-tetrahydrocannabinol; Mmp23/CA-MMP, matrix metalloproteinase 23/cysteine array matrix metalloproteinase.

* Corresponding author at: 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. Tel.: +972 8 934 4539; fax: +972 8 934 4131.
E-mail address: zvi.vogel@weizmann.ac.il (Z. Vogel).

0197-0186/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2011.12.002

affinity for both CB_1 and CB_2 receptors and as stated above, is devoid of the unwanted psychotropic effects characteristic of marijuana or THC (Pertwee, 2005; Mechoulam et al., 2007; Izzo et al., 2009). CBD displays a diversity of actions, including anticonvulsive, sedative, hypnotic, antipsychotic, anti-inflammatory and neuroprotective properties (Mechoulam et al., 2002, 2007; Scuderi et al., 2009; Liu et al., 2010; Kozela et al., 2010, 2011; Juknat et al., 2011). As CBD is not an efficient ligand of either CB_1 or CB_2, these effects are probably mediated through other receptors/targets (see below).

A wide range of literature reports the effects of phytocannabinoids on various populations of immune cells (Klein et al., 1998; Cabral and Staab, 2005; Croxford and Yamamura, 2005; Kozela et al., 2010, 2011; Juknat et al., 2011; Rimmerman et al., 2011). Both THC and CBD have been shown to decrease cytokine production in human immune cell lines (Srivastava et al., 1998; Kozela et al., 2010) and to suppress T cell proliferation and their effector functions (Nahas et al., 1974; Cabral and Dove Pettit, 1998; Kaplan et al., 2003; Klein et al., 2004; Jan et al., 2007; Kozela et al., 2011). However, the molecular mechanisms involved in these cannabinoid-mediated effects are not yet fully characterized. Altered adenosine signaling through inhibition of its uptake, has been reported as a potential non-cannabinoid receptor mechanism by which CBD, and less so THC, can decrease inflammation (Carrier et al., 2006). Other studies identified PPAR-γ as an intracellular target, which mediates the cannabinoid-associated immunosuppression in a manner that is independent of CB_1 and CB_2 receptors (O'Sullivan, 2007). Other targets including the G-protein-coupled receptors GPR55 and GPR18 as well as the transient receptor potential (TRP) channels were also suggested (Stella, 2010; De Petrocellis and Di Marzo, 2010; Pertwee et al., 2010).

Among the immune cells, microglia are considered to be the resident macrophage-like cells of the central nervous system (CNS). These cells are known to exert an important role in brain's innate immunity and in inflammatory neuropathologies (Hanisch and Kettenmann, 2007; Graeber and Streit, 2010). Microglia can be activated by injury or infection and have been suggested to be the first line of defense in the CNS (Streit, 2005). Microglial activation is associated with production and secretion of a variety of compounds such as cytokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), matrix metalloproteinases and prostaglandins. Although acute microglial activation is a protective mechanism involved in regulating tissue repair and recovery, excessive or chronic activation can lead to harmful effects (Hanisch and Kettenmann, 2007). Interestingly, the mechanisms that give rise to the protective or the damaging microglial phenotypes are not fully elucidated. The possibilities of enhancing microglial-mediated innate immunity in the brain and of preventing the harmful effects associated with their chronic activation could offer new therapeutic approaches to the treatment of brain injury and neurodegenerative diseases.

Several groups including ours, have shown that microglial cells express CB_1 and CB_2 receptors (Pietr et al., 2009; for review see Stella (2010)). However, in addition to these receptors, cannabinoids were shown to affect microglial cells activation via various CB_1/CB_2 independent mechanisms (Walter and Stella, 2004; Stella, 2010). Recently, using the murine microglial BV-2 cell line, our group reported that the cannabinoids THC and CBD differentially inhibit the lipopolysaccharide (LPS)-activated NF-κB and IFN-β/STAT proinflammatory pathways in a CB_1/CB_2 independent manner (Kozela et al., 2010) and that CBD affects the expression of genes involved in oxidative stress, glutathione deprivation and inflammation (Juknat et al., 2011). Moreover, several of the genes affected seem to be related to zinc homeostasis.

1.2. Zinc homeostasis

Zinc is a critical factor in the regulation of a large number of genes via its role in the activation of transcription factors, as well as by promoting the link between zinc finger proteins and DNA. Cytosolic free zinc concentration is tightly controlled by zinc-binding proteins which include zinc sensors, zinc transporters and zinc buffering proteins (such as the metallothioneins; Mts). In mammalian cells, zinc transport from the cytoplasm toward the lumen of intracellular organelles or to the outside of the cell are mediated by the ZnT/SlcC30 family of transporters. There are at least 10 members of this ZnT family, most of them are ubiquitously expressed and their expression is tightly and dynamically coupled to changes in the intracellular levels of zinc.

Within the cytoplasm, zinc is bound to metal-free apo-metallothionein (apo-Mt) and to protonated glutathione to generate Zn–Mt and G-SZn, respectively. In mammalians there are four metallothionein (Mt) isoforms. Mt1 and Mt2 are ubiquitously expressed in virtually all mammalian cells, of which Mt2a is the major representative. Mt3 is expressed predominantly in the brain and Mt4 is limited to epithelial cells in skin and tongue (see Haq et al. (2003) for review). Mts belong to a family of low molecular weight, cysteine-rich, and high metal containing (as metal-thiolate complex clusters) stress response proteins, involved in a broad range of functions, including zinc and copper homeostasis, heavy metal (Hg, Cd, Ag, Cu) detoxification, scavenging of ROS (decreasing $OH^{\cdot}$ radicals), immune defense responses, regulation of Zn fingers and Zn-containing transcription factors, protein–protein and protein–nucleotide interactions, as well as cell survival and differentiation (for reviews, see Andrews (2000) and Haq et al. (2003)). Some of these actions seem to have physiological relevance in a range of chronic neurological disorders, in which inflammation and oxidative stress are central to the pathophysiology (Penkowa, 2006; Haase and Rink, 2009, and references therein). Many studies have by now demonstrated that the Mts are multipurpose factors important for host defense responses, immunoregulation, cell survival and brain repair. Moreover, it was reported that both Mt1 and Mt2 may reduce inflammation by interfering directly with immune cell–cell interactions, as both Mts were demonstrated to bind specifically to the membranes of activated macrophages, T and B cells, thus, inactivating them and decreasing the immune response (Penkowa, 2006, and references therein).

As described above, cannabinoids were shown to affect the immune system and to posses anti-inflammatory effects. However, no information has been yet reported regarding the effects of cannabinoids on the expression of zinc dependent genes. In this paper we describe the effects of CBD and THC on the expression of these genes.

2. Results

2.1. Effect of CBD on gene expression

mRNA samples were prepared from BV-2 cells treated for 6 h with CBD or THC (both at 10 μM) or with vehicle (ethanol 0.1%) as previously described (Juknat et al., 2011). Characterization of the transcriptional effects of CBD and THC were performed through comparative microarray analysis using the Illumina MouseRef-8 BeadChip platform. Differentially expressed genes were classified according to their gene ontology (GO), using DAVID Bioinformatics online tools (*D*atabase for *A*nnotation, *V*isualization and *I*ntegrated *D*iscovery; http://david.abcc.ncifcrf.gov/; Huang et al., 2009) and the *I*ngenuity *P*athway *A*nalysis (*IPA*; Ingenuity® Systems, http://www.ingenuity.com/). The *DAVID* tool uses the biological knowledge accumulated in public databases and provides a

comprehensive set of enriched functional annotation tools to understand the biological meaning behind a large list of genes. *DAVID* provides a structured and controlled vocabulary (ontologies) for describing the roles of genes and gene products. Three ontologies were developed to describe the roles of the gene products. These include the associated biological processes, molecular functions and cellular components (http://www.geneontology.org/; Ashburner et al., 2000).

The analysis of the Illumina gene array (containing 24,000 gene probes) revealed that CBD affected the expression of many genes. Gene transcripts (1204) were significantly regulated by CBD (680 were upregulated and 524 downregulated). From these genes, 123 transcripts were upregulated and 38 genes downregulated by 2-fold or more ($p \leqslant 0.005$; Juknat et al., 2011). *DAVID* analysis of the gene products that were significantly upregulated by CBD (Fig. 1) showed genes related to amino acid metabolism and glutathione activity, tRNA aminoacylation, molecular and ion transport as well as oxidative stress response. The CBD-downregulated genes involve various subsets of genes associated with regulation of cell cycle, DNA replication and RNA processing as well as biosynthetic processes and gene expression (Fig. 1).

Contrary to the large number of genes affected by CBD, a much smaller number was found to be affected by THC. From the total transcripts, 94 genes were significantly modulated by THC (58 were upregulated and 36 were downregulated). From these genes, 8 were upregulated by THC by 2-fold or more ($p \leqslant 0.005$) and none were downregulated to this extent. *DAVID* analysis of all the genes that were significantly affected by THC is shown in Fig. 2. It shows that upregulated genes were mainly related to transmembrane receptor and transporter activities as well as to cellular components integral to the membrane.

The *DAVID* analysis of the CBD-upregulated transcripts shows genes belonging to the following *Enriched Functional Annotations:* transmembrane transporter activity, inorganic cation transporter activity and response to oxidative stress. According to this result and after performing gene-by-gene inspection, we found that CBD affects the expression of various genes that are known to be involved in zinc homeostasis. Table 1 shows that CBD, but not THC, upregulates the expression of *metallothionein 2* (*Mt2*; by 7.9-fold), *N-myc-downstream regulated gene 1* (*Ndrg1*; 6.5-fold), *matrix metalloproteinase 23* (*Mmp23*; 2.5-fold) as well as the zinc transporters *ZnT1/Slc30a1* (*Solute carrier family 30, member 1*; 2.6-fold) and *Zip4/Slc39a4* (*Solute carrier family 39, member 4*; 3.5-fold) but downregulates *Zip10/Slc39a10 (Solute carrier family 39, member 10*; by 52%) and *zinc finger protein 472* (*Zfp472* by 80%). Quantitative real time reverse transcription polymerase chain reaction (qPCR), using *β2-microglobulin* (*B2m*) as a reference gene, validated these results (see Table 1). The primer sets used for the qPCR validation are shown in Table 2.

The zinc transporter ZnT1/Slc30a1 (that as described above is upregulated by CBD) is located at the plasma membrane and is the primary regulator of cellular zinc efflux (Eide, 2006). Immunohistochemical analysis demonstrated endogenous ZnT1/Slc30a1 expression in cultured astrocytes, microglia and oligodendrocytes (Nolte et al., 2004). It has been implicated in neuroprotection against toxic zinc in cultured neurons and astroglia, and its expression is upregulated in brains of rodents undergoing seizures or ischemia (Nolte et al., 2004; Sensi et al., 2009). In animal models of cerebral ischemia, nitric oxide triggers the intraneural release of zinc, that in turn upregulates the expression of ZnT1/Slc30a1 as a neuroprotective action. Results obtained from our gene array analysis showed that from the various members of the ZnT/Slc30a family, BV-2 cells also express *ZnT4, ZnT5, ZnT6, ZnT7* and *ZnT8* mRNAs, but the expression of these genes were not markedly affected by either CBD or THC.

In mammalian cells, zinc from the extracellular space and from intracellular organelles enters the cytoplasm through specialized transmembrane proteins of the ZIP/Slc39 family, through a process facilitated by HCO_3^- or H^+ gradients (Eide, 2006; Sensi et al., 2009). ZIP transporters are ubiquitously expressed in the brain and peripheral tissues. The membrane levels and distribution of these transporters is regulated by intracellular zinc, undergoing rapid endocytosis and degradation in response to increases in intracellular zinc. Here we show that CBD upregulates *Zip4*/*Slc39a4* (3.5-fold) but downregulates *Zip10/Slc39a10* (by 52%). The expression of these two zinc transporters which are known to have antagonistic functions, is controlled by MTF-1 (metal responsive element (MRE)-binding transcription factor-1), a transcription factor that can act as an activator of *Zip4*/*Slc39a4* and as a repressor of *Zip10*/*Slc39a10*. As for the other members of the ZIP/Slc39 family of transporters, gene array analysis showed that BV-2 microglial cells express *Zip1–3, Zip5–6, Zip8–9* and *Zip14* mRNAs but the expression of these genes were not affected by either CBD or THC treatments.

Another MTF-1 target gene found to be affected by CBD, is the *Ndrg1* (upregulated by 6.5-fold). *Ndrg1* has been shown to be induced by different physiological and stress conditions (Melote et al., 2010). Ndrg1 has been reported to be involved in inflammation and to play a central role in allergy, anaphylaxis, wound healing and defense against pathogens. Ndrg1 is expressed in oligodendrocytes and is localized to the cytoplasm of myelinating Schwann cells. Ndrg1-deficient mice exhibit progressive demyelination in peripheral nerves, suggesting that this deficiency is a primary cause of Schwann cell dysfunction (Melote et al., 2010, and references therein). Thus, the increase in Ndrg1 expression by CBD could have an important role in the CBD anti-inflammatory response and the capacity of CBD to ameliorate the symptoms of the myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice (Kozela et al., 2011).

The most upregulated gene by CBD is the MTF-1 target gene *Mt2* (7.9-fold). The Mt proteins were found to be increased in response to CNS damage, including ischemia and spinal cord injury, as well as in neurodegenerative diseases such as Alzheimer's disease. Moreover, Mt was reported to have protective effects due to its ROS scavenging properties and its capacity to reduce the inflammatory response associated with CNS injury, thus leading to enhanced recovery (Penkowa and Hidalgo, 2000, 2001; Penkowa, 2006).

3. Discussion

Regulation of gene expression by metal ions has been reported from bacteria to mammalians. Metal ions regulate genes that are involved in protection against metal toxicity as well as genes involved in the homeostasis of essential metals. Several transcription factors which interact with metal ions serve as cellular metal sensors that regulate gene transcription as well as mRNA translation and stability.

Zinc homeostasis as well as cellular responses to heavy metals are known to be regulated by the zinc-sensor transcription factor MTF1. MTF-1 is a zinc-dependent transcriptional activator that controls the expression of the various *Mts* and of the zinc efflux transporter *ZnT1/Slc30a1* (see Lichten et al. (2001), Laity and Andrews (2007) and Jackson et al. (2008) for reviews). Interestingly, MTF-1 can serve as both activator or repressor, depending on the target gene and zinc concentration (Wimmer et al., 2005). Thus, MTF-1 controls the expression of two zinc transporters with antagonistic functions, namely *ZnT1/Slc30a1* and *Zip10*/*Slc39a10*. The latter also includes MREs in its upstream region. Indeed, as shown in Section 2, both genes were found to be affected by CBD but in inverse directions.

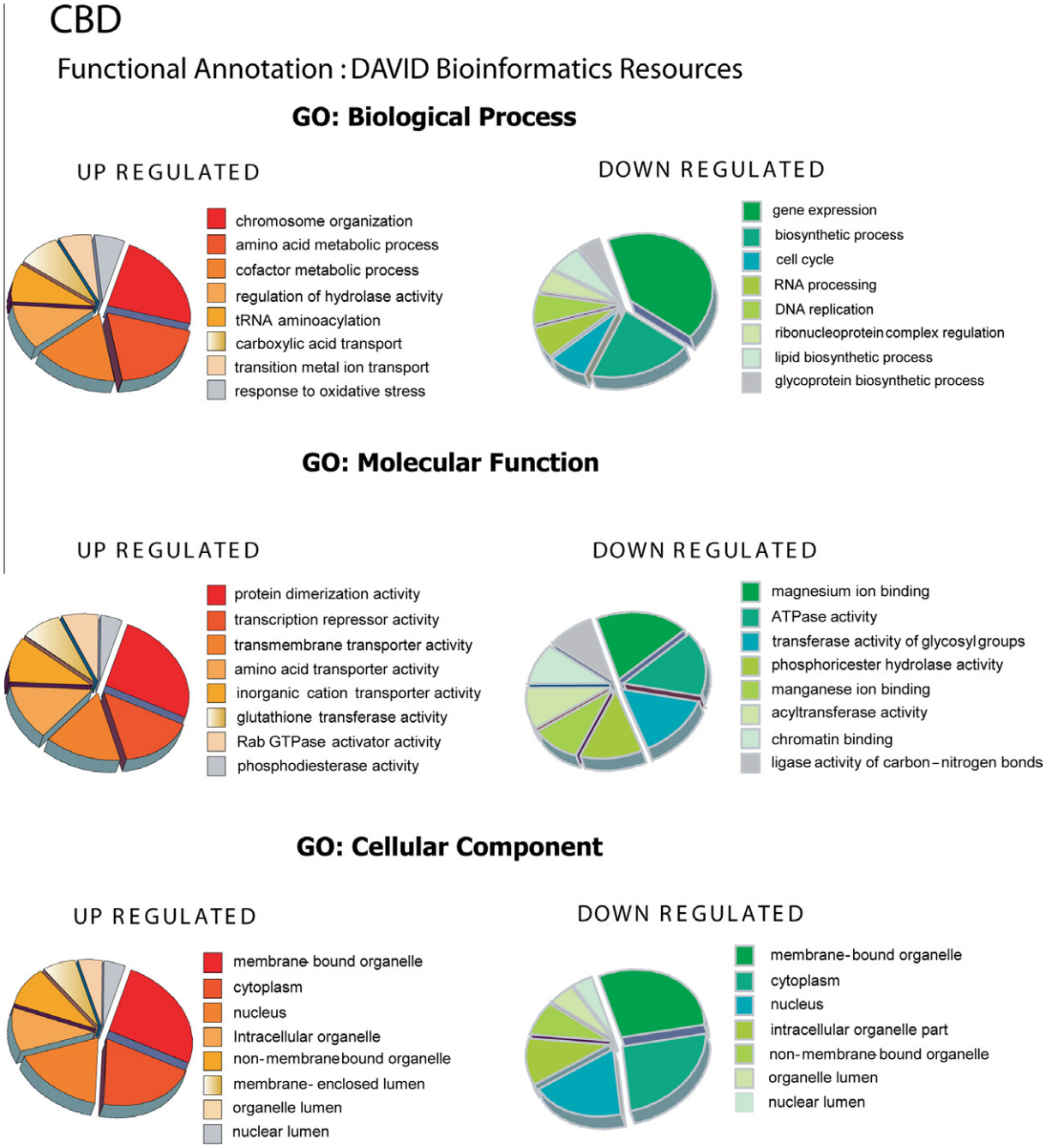

Fig. 1. Distribution of differentially expressed gene products following CBD treatment, using ontology functional annotation. The significant transcriptomes were uploaded to DAVID Bioinformatic Resources where the Gene Functional Classification tool was applied to generate clusters of functionally related genes. The Functional Annotation Clustering tool was used to generate clusters of GO terms. The GO analysis was performed separately for up- (red) and down-regulated (green) gene products (at $p < 0.05$). The top pie chart panel represents the ontology Biological Processes, the middle panel represents the ontology Molecular Functions and the bottom pie chart represents the ontology Cellular Components.

Besides coping with heavy metal toxic levels, MTF-1 also mediates induction of genes in response to other type of stress situations, such as oxidative stress and hypoxia, implying a more general role for this transcription factor in cellular stress response (Wimmer et al., 2005, and references therein). For example, the glutamate–cysteine ligase catalytic subunit (GCLC), the rate-limiting enzyme in glutathione biosynthesis, is regulated by MTF-1 in a metal-inducible manner (Lichten et al., 2001; Laity and Andrews, 2007; Jackson et al., 2008). However, the molecular mechanism(s) by which MTF-1 is activated in response to different stress stimuli (by triggering its DNA binding and subsequent transactivation of the target genes), remains to be elucidated.

Matrix metalloproteinases (Mmps) belong to a large family of zinc-dependent endopeptidases involved in the proteolytic degradation of extracellular matrix components. Mmps regulate many physiological and pathological processes, such as differentiation, proliferation, angiogenesis and apoptosis. Some of these functions are mediated by the ability of Mmps to catalyze the hydrolysis of a variety of substrates including membrane-bound precursors of cytokines, growth factors or hormone receptors. Moreover, Mmps increase the permeability of the blood–brain barrier as part of the neuroinflammatory response in hypoxia–ischemia, multiple sclerosis (MS) and infection (Rosenberg, 2009, and references therein). Mmps are kept inactive by interaction between a cys-

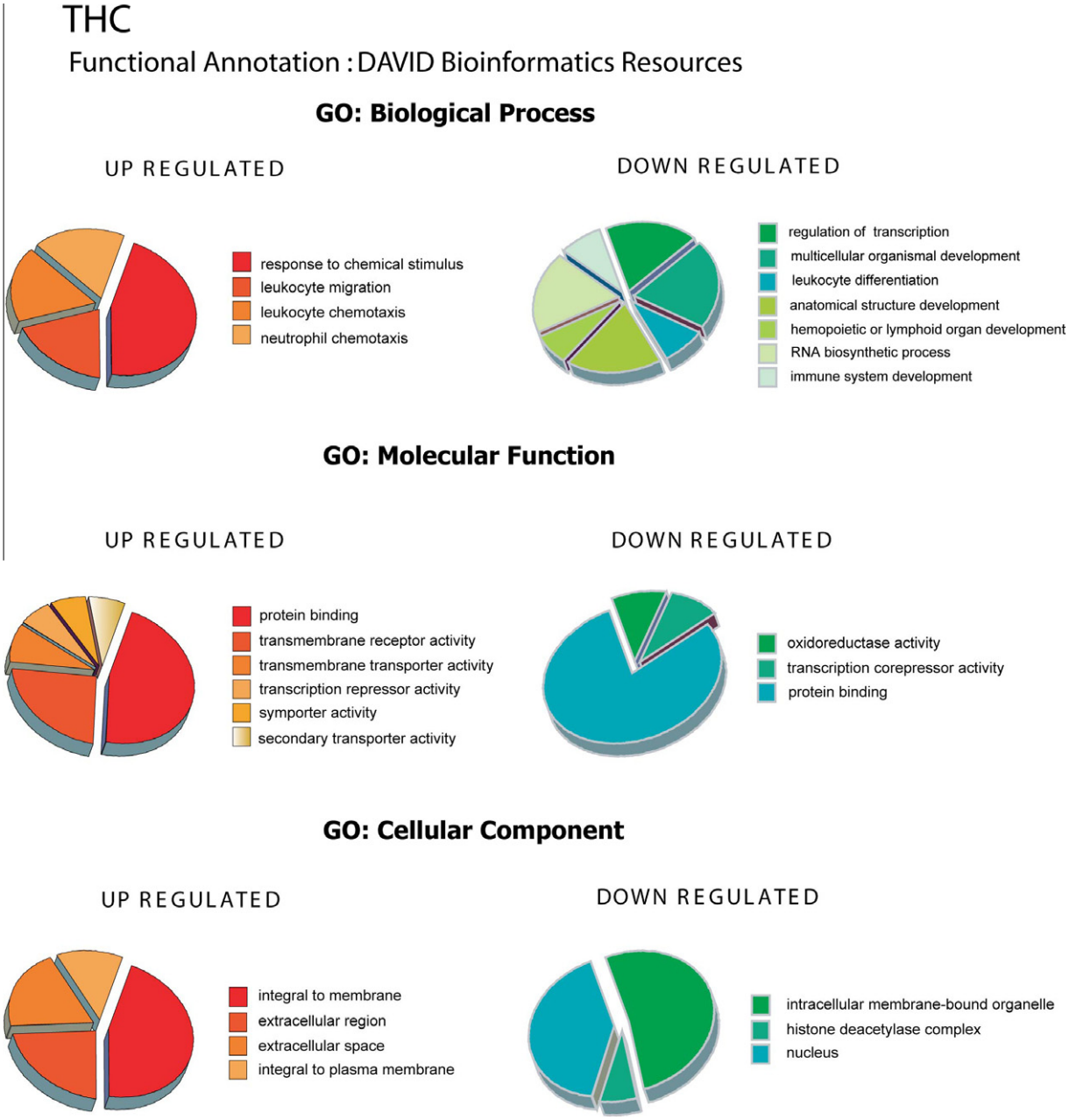

Fig. 2. Distribution of differentially expressed gene products following THC treatment, using ontology functional annotation. Gene ontology (GO) analysis was performed for up- (red) and down-regulated (green) gene products. Details are as indicated in Fig. 1.

teine–sulphydryl group in the propeptide domain and the zinc bound to the catalytic domain; and their activation requires proteolytic removal of the propeptide prodomain. An exception to this group is the Mmp 23 (also known as cysteine array matrix metalloproteinase, CA-MMP), shown here to be upregulated by CBD (2.5-fold; Table 1). Structurally, Mmp 23/CA-MMP lacks the classical cysteine switch, but it brings two novel functional domains to the repertoire of Mmps, a distinct cysteine array (CA) and a novel Ig-like domain, suggesting that CA-MMP may employ an entirely different mechanism for latency because there is no cysteine in its putative prodomain. Alignment of the catalytic subunit with other Mmps, indicates strong homology surrounding the zinc binding catalytic site. Mmp 23/CA-MMP is localized as a type II transmembrane proteinase via a transmembrane anchor revealed as an N-terminal hydrophobic segment of 17 residues. A specific cleavage in Arg^{79} releases the active enzyme to the extracellular milieu, showing that Mmp 23/CA-MMP can be regulated by a single proteolytic cleavage for both activation and secretion. Mouse Mmp 23/CA-MMP is expressed at high level in heart, lung and spleen tissues of mouse, but its physiological function is presently unknown (Pei, 1999; Pei et al., 2000). Mmps are known to regulate inflammatory processes by proteolytic cleavage of proinflammatory cytokines (Van Lint and Libert, 2007). Several lines of evidence show that Mmps can either promote or repress inflammation by the direct proteolytic processing of inflammatory cytokines and chemokines to activate, inactivate, or antagonize chemokine function (see Manicone and McGuire (2008) for review). These effects of Mmp23/CA-MMP and the fact that CBD upregulates the transcription of Mmp23/CA-MMP is in line with the anti-inflammatory effects exerted by CBD.

Table 1
Effect of CBD and THC on genes involved in zinc homeostasis in BV-2 microglial cells.

Gene name	Description	Accession No.	Gene array results (values obtained versus control = 1)		qPCR (values obtained versus control = 1)[a]	
			CBD	THC	CBD	THC
Stress response						
Mt2	Metallothionein 2	NM_008630.2	7.9	1.3	5.3 ± 0.5	1.1 ± 0.3
Ndrg1	N-myc downstream regulated gene 1	NM_008681.2	6.5	1.2	3.7 ± 0.3	1.6 ± 0.1
Membrane transport						
Slc39a4	Solute carrier family 39 (zinc transporter), member 4	NM_028064.2	3.5	1.6	3.8 ± 0.4	1.4 ± 0.6
Slc30a1	Solute carrier family 30 (zinc transporter), member 1	NM_009579.2	2.6	1.0	2.1 ± 0.3	0.9 ± 0.1
Slc39a10	Solute carrier family 39 (zinc transporter), member 10	NM_172653.2	0.48	0.83	0.16 ± 0.04	0.9 ± 0.1
Regulation of transcription						
Zfp472	Zinc finger protein 472	NM_153063.3	0.20	1.2	0.6 ± 0.1	0.9 ± 0.1
Adhesion and migration						
Mmp23	Matrix metalloproteinase 23	NM_011985.2	2.5	1.2	2.6 ± 0.7	1.2 ± 0.2

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

Zinc is involved in many cellular functions under both normal and pathological conditions. It is absolutely required for cellular development and survival (MacDonald, 2000); and accordingly, zinc deficiency causes developmental anomalies in humans and animals, immune dysfunction, neurological problems as well as increased mortality (Sensi et al., 2009). On the other side, increased levels of free zinc is highly cytotoxic. Zinc toxicity (by high intracellular levels) could arise from unfavorable competition for binding sites with other metals (*e.g.*, in enzymes and metal ion transport proteins) as well as from depletion of glutathione, leading to oxidative stress (Laity and Andrews, 2007, and references therein). Therefore, maintaining the appropriate zinc level is of critical importance for normal cell function. Zinc homeostasis is coordinated via regulation by proteins involved in its uptake and efflux as well as mobilization and intracellular storage. Here we show that CBD treatment upregulates the mRNAs for *Slc30a1* (*ZnT1*; involved in efflux of zinc), *Slc39a4* (*ZIP4*; involved in uptake of zinc) and *Mt2* (an important zinc buffer protein) and downregulates *Slc39a10* (*ZIP10*; involved in uptake of zinc). All of these molecules serve as key regulators in maintaining zinc homeostasis. The changes elicited by CBD in the expression of *Slc30a1*, *Slc39a4, Slc39a10* and *Mt2* mRNA's are consistent with increased availability of intracellular zinc in CBD-treated BV-2 microglial cells. In this regard, there are reports that show that zinc decreases oxidative stress, as zinc is an inhibitor of NADPH oxidase (which catalyzes the production of $O_2^{\cdot-}$ from O_2, using NADPH as electron donor), and serves as a cofactor of the zinc/copper superoxide dismutase (which catalyzes dismutation of $O_2^{\cdot-}$ to H_2O_2) and induces the synthesis of Mts (an scavenger of $OH^{\cdot}$ radical) (Prasad, 2008, 2009). On the other hand, a number of studies with various cell culture models suggest that high intracellular zinc concentration may lead to increased generation of ROS (Song et al., 2004; Kindermann et al., 2005). Mt2 which buffers zinc is scavenging ROS due to its high cysteine content. Thus, the increased Mt2 expression observed following CBD treatment could represent a mechanism for cellular protection from ROS formation.

Zinc seems to play a very important and critical role in the functions of T cells. Experimental human models (obtained by restricting dietary daily zinc intake) suggest that cell-mediated immune dysfunctions in human zinc deficiency may be due to an imbalance between T helper 1 and T helper 2 cell functions (Prasad, 2008, 2009). EAE is an autoimmune animal model for the human disease MS. MS is clinically characterized by paralysis and histopathological presence of immune infiltrates, as well as by strong expression of proinflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). Macrophages and lymphocytes seem to be the main source of these proinflammatory cytokines, all of which carry the potential to exacerbate the disease.

Table 2
Primer sequences used for qPCR.

		Sequence (5′ → 3′)	Length (bp)[a]	Accession No.[b]
B2mg	FW[c]	ATG GGA AGC CGA ACA TAC TG	176	NM_009735.3
	RV	CAG TCT CAG TGG GGG TGA AT		
Mt2	FW	CAT CTG CAA AGA GGC TTC CGA CAA	133	NM_008681.2
	RV	AAG TTG TGG AGA ACG AGT CAG GGT		
Ndrg1	FW	TGC TAG AAC CTG GTG GGT GTG ATT	133	NM_008681.2
	RV	AGC GAT GGG CCA GTT AAG AGA CAT		
Slc39a4	FW	CCT TTA CGT GGC GCT TTG TGA CAT	200	NM_028064.2
	RV	AGC AGA GTC AAC AGA CAG GGA CAA		
Slc30a1	FW	TCT GCC TTG AAA CAG CAC AAT GGG	138	NM_009579.3
	RV	TTG CCG CAC TCG AGT TCA AGA TGA		
Slc39a10	FW	TTC GCC ATC ATG CTG GTT ATT GCC	140	NM_172653.2
	RV	AGC GAC GTG CAT ACA GTA CAA GGA		
Mmp23	FW	TCA TTG CCA ACG CAG TCA ACG AAG	108	NM_011985.2
	RV	AGC AGT GTC AAT TCC TCA CTC GGA		
Zfp472	FW	TGG GAA AGC CTT CAT CCA ACG TGA	198	NM_153063.3
	RV	AGG TAT GTG GAA CAG GTG AAC GCT		

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

Furthermore, macrophages are the major producers of ROS, known to be elevated during EAE and MS. It was shown that during CNS inflammation, microglia/macrophages as well as reactive astrocytes increase the tissue protective factors metallothioneins Mt1 and Mt2 (Ashner, 1996; Penkowa and Hidalgo, 2000, and references therein). Penkowa and Hidalgo (2000) showed that the expression of Mt1/Mt2 is significantly increased by myelin basic protein-induced EAE in various CNS areas of the rat, including brain stem, cerebellum and spinal cord. In addition, they reported that i.p. administration of zinc–Mt2 complex significantly reduced the clinical symptoms and histopathological signs of EAE, as evident by decreased macrophages and T cells infiltration. Moreover, the zinc–Mt2 treatment significantly reduced oxidative stress (measured as immunoreactivity of malondialdehyde, inducible nitric oxide synthase and nitrotyrosine), reduced the proinflammatory cytokines IL-6 and TNF-α in EAE as well as decreased apoptotic cell death of neurons and oligodendrocytes in the brain stem, cerebellum and spinal cord (Penkowa and Hidalgo, 2001). In our previous work, we reported that treatment with CBD ameliorates the severity of the clinical signs of MOG-induced EAE in C57BL/6 mice. This effect of CBD was accompanied by diminished axonal damage and inflammation as well as reduced microglial activation and T cell recruitment in the spinal cord of the MOG-injected mice. Furthermore, CBD inhibited MOG-induced T cell proliferation *in vitro* (Kozela et al., 2011). These observations suggest that CBD has a potential for alleviating MS-like pathology. At this stage, it is not yet clear whether the effect of CBD in upregulating Mt2 is responsible for its effect in ameliorating the EAE disease symptoms.

In conclusion, we show here that CBD affects the expression of several genes involved in zinc homeostasis. This effect of CBD on zinc regulation is in line with the antioxidant and anti-inflammatory effects of this cannabinoid.

Conflict of interest

The authors report no conflicts of interest.

Acknowledgments

This work was supported by the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation and by the Dr. Miriam and Sheldon G. Adelson Center for the Biology of Addictive Diseases. A.J. is supported by the Israeli Ministry for Absorption in Science.

References

Andrews, G.K., 2000. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem. Pharmacol. 59, 95–104.
Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M., Sherlock, G., 2000. Gene ontology: tool for the unification of biology. Nat. Genet. 25, 25–29.
Ashner, M., 1996. The functional significance of brain metallothioneins. FASEB J. 10, 1129–1136.
Cabral, G.A., Dove Pettit, D.A., 1998. Drugs and immunity: cannabinoids and their role in decreased resistance to infectious disease. J. Neuroimmunol. 83, 116–123.
Cabral, G.A., Griffin-Thomas, L., 2009. Emerging role of the cannabinoid receptor CB_2 in immune regulation: therapeutic prospects for neuroinflammation. Expert Rev. 11, e3.
Cabral, G.A., Staab, A., 2005. Effects on the immune system. Handb. Exp. Pharmacol. 168, 385–423.
Cabral, G.A., Raborn, E.S., Griffin, L., Dennis, J., Marciano-Cabral, F., 2008. CB2 receptors in the brain: role in central immune function. Br. J. Pharmacol. 153, 240–251.
Carrier, E.J., Auchampach, J.A., Hillard, C.J., 2006. Inhibition of an equilibrative nucleoside transporter by cannabidiol: a mechanism of cannabinoid immunosuppression. Proc. Natl. Acad. Sci. USA 103, 7895–7900.
Croxford, J.L., Yamamura, T., 2005. Cannabinoids and the immune system: potential for the treatment of inflammatory diseases? J. Neuroimmunol. 166, 3–18.
De Petrocellis, L., Di Marzo, V., 2010. Non-CB1, non-CB2 receptors for endocannabinoids, plant cannabinoids and synthetic cannabimimetics: focus on G-protein-coupled receptors and transient receptor potential channels. J. Neuroimmune Pharmacol. 5, 103–121.
Earleywine, M., 2002. Understanding Marijuana: A New Look at the Scientific Evidence. Oxford University Press.
Eide, D.J., 2006. Zinc transporters and the cellular trafficking of zinc. Biochim. Biophys. Acta 1763, 711–722.
Galve-Roperh, I., Sanchez, C., Cortes, M.L., del Pulgar, T.G., Izquierdo, M., Guzman, M., 2000. Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. Nat. Med. 6, 313–319.
Graeber, M.B., Streit, W.J., 2010. Microglia: biology and pathology. Acta Neuropathol. 119, 89–105.
Haase, H., Rink, L., 2009. Functional significance of zinc-related signaling pathways in immune cells. Annu. Rev. Nutr. 29, 133–152.
Hanisch, U.K., Kettenmann, H., 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat. Neurosci. 10, 1387–1394.
Haq, F., Mahoney, M., Koropatnick, J., 2003. Signaling events for metallothionein induction. Mutat. Res. 533, 211–226.
Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57.
Izzo, A.A., Borrelli, F., Capasso, R., Di Marzo, V., Mechoulam, R., 2009. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. Trends Pharmacol. Sci. 30, 515–527.
Jackson, K.A., Valentine, R.A., Coneyworth, L.J., Mathers, J.C., Ford, D., 2008. Mechanisms of mammalian zinc-regulated gene expression. Biochem. Soc. Trans. 36, 1262–1266.
Jan, T.R., Su, S.T., Wu, H.Y., Liao, M.H., 2007. Suppressive effects of cannabidiol on antigen-specific antibody production and functional activity of splenocytes in ovalbumin-sensitized BALB/c mice. Int. Immunopharmacol. 7, 773–780.
Juknat, A., Pietr, M., Kozela, E., Rimmerman, N., Levy, L., Coppola, G., Geschwind, D., Vogel, Z., 2011. Differential transcriptional profiles mediated by exposure to the cannabinoids cannabidiol and Δ^9-tetrahydrocannabinol in BV-2 microglial cells. Br. J. Pharmacol. doi:10.1111/j.1476-5381.2011.01461.x.
Kaplan, B.L., Rockwell, C.E., Kaminski, N.E., 2003. Evidence for cannabinoid receptor-dependent and -independent mechanisms of action in leukocytes. J. Pharmacol. Exp. Ther. 306, 1077–1085.
Kindermann, B., Doering, F., Fuchs, D., Pfaffl, M.W., Daniel, H., 2005. Effects of increased cellular zinc levels on gene and protein expression in HT-29 cells. BioMetals 18, 243–253.
Klein, T.W., Cabral, G.A., 2006. Cannabinoid-induced immune suppression and modulation of antigen-presenting cells. J. Neuroimmune Pharmacol. 1, 50–64.
Klein, T.W., Newton, C., Friedman, H., 1998. Cannabinoid receptors and immunity. Immunol. Today 19, 373–381.
Klein, T.W., Newton, C., Larsen, K., Chou, J., Perkins, I., Lu, L., Nong, L., Friedman, H., 2004. Cannabinoid receptors and T helper cells. J. Neuroimmunol. 147, 91–94.
Kogan, N.M., Mechoulam, R., 2007. Cannabinoids in health and disease. Dialogues Clin. Neurosci. 9, 413–430.
Kozela, E., Pietr, M., Juknat, A., Rimmerman, N., Levy, R., Vogel, Z., 2010. Cannabinoids Δ^9-tetrahydrocannabinol and cannabidiol differentially inhibit the LPS-activated NF-κB and IFNβ/STAT proinflammatory pathways in BV-2 microglial cells. J. Biol. Chem. 285, 1616–1625.
Kozela, E., Lev, N., Kaushansky, N., Eilam, R., Rimmerman, N., Levy, R., Ben-Nun, A., Juknat, A., Vogel, Z., 2011. Cannabidiol inhibits pathogenic T-cells, decreased spinal microglial activation and ameliorates multiple sclerosis-like disease in mice. Br. J. Pharmacol. 163, 1507–1519.
Laity, J.H., Andrews, G.K., 2007. Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1). Arch. Biochem. Biophys. 463, 201–210.
Le Foll, B., Goldberg, S.R., 2005. Cannabinoid CB_1 receptor antagonists as promising new medications for drug dependence. J. Pharmacol. Exp. Ther. 312, 875–883.
Lichten, P., Wang, Y., Belser, T., Georgiev, O., Certa, U., Sack, R., Schaffner, W., 2001. Target gene search for the metal-responsive transcription factor MTF-1. Nucleic Acids Res. 29, 1514–1523.
Liu, D.Z., Hu, C.M., Huang, C.H., Wey, S.P., Jan, T.R., 2010. Cannabidiol attenuates delayed-type hypersensitivity reactions via suppressing T-cell and macrophage reactivity. Acta Pharmacol. Sin. 31, 1611–1617.
MacDonald, R.S., 2000. The role of zinc in growth and cell proliferation. J. Nutr. 130, 1500S–1508S.
Manicone, A.M., McGuire, J.K., 2008. Matrix metalloproteinases as modulators of inflammation. Semin. Cell Dev. Biol. 19, 34–41.
Massi, P., Vaccani, A., Bianchessi, S., Costa, B., Macchi, P., Parolaro, D., 2006. The non-psychoactive cannabidiol triggers caspase activation and oxidative stress in human glioma cells. Cell. Mol. Life Sci. 63, 2057–2066.
McKallip, R.J., Lombard, C., Martin, B.R., Nagarkatti, M., Nagarkatti, P.S., 2002. Delta(9)-tetrahydrocannabinol-induced apoptosis in the thymus and spleen as a mechanism of immunosuppression in vitro and in vivo. J. Pharmacol. Exp. Ther. 302, 451–465.
Mechoulam, R., Parker, L.A., Gallily, R., 2002. Cannabidiol: an overview of some pharmacological aspects. J. Clin. Pharmacol. 42, 11S–19S.
Mechoulam, R., Peters, M., Murillo-Rodriguez, E., Hanus, L.O., 2007. Cannabidiol-recent advances. Chem. Biodiversity 4, 1678–1692.

Melote, V., Qu, X., Ongenaert, M., van Criekinge, W., de Bruine, A.P., Scott Baldwin, H., van Engeland, M., 2010. The N-myc downstream regulated gene (NDRG) family: diverse functions, multiple applications. FASEB J. 24, 4153–4166.

Murray, R.M., Morrison, P.D., Henquet, C., Di Forti, M., 2007. Cannabis, the mind and society: the hash realities. Nat. Rev. Neurosci. 8, 885–895.

Nahas, G.G., Suciu-Foca, N., Armand, J.P., Morishima, A., 1974. Inhibition of cellular mediated immunity in marihuana smokers. Science 183, 419–420.

Nolte, C., Gore, A., Sekler, I., Kresse, W., Hershfinkel, M., Hoffmann, A., Kettenmann, H., Moran, A., 2004. ZnT-1 expression in astroglial cells protects against zinc toxicity and slows the accumulation of intracellular zinc. Glia 48, 145–155.

O'Sullivan, S.E., 2007. Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. Br. J. Pharmacol. 152, 576–582.

Pei, D., 1999. CA-MMP: a matrix metalloproteinase with a novel cysteine array, but without the classic cysteine switch. FEBS Lett. 457, 262–270.

Pei, D., Kang, T., Qi, H., 2000. Cysteine Array Matrix Metalloproteinase (CA-MMP)/MMP-23 is a type II transmembrane matrix metalloproteinase regulated by a single cleavage for both secretion and activation. J. Biol. Chem. 275, 33988–33997.

Penkowa, M., 2006. Metallothioneins are multipurpose neuroprotectans during brain pathology. FEBS J. 273, 1857–1870.

Penkowa, M., Hidalgo, J., 2000. Metallothionein I and II expression and their role in experimental autoimmune encephalomyelitis. Glia 32, 247–263.

Penkowa, M., Hidalgo, J., 2001. Metallothionein treatment reduces proinflammatory cytokines IL-6 and TNF-α and apoptotic cell death during experimental autoimmune encephalomyelitis (EAE). Exp. Neurol. 170, 1–14.

Pertwee, R.G., 2005. Pharmacological actions of cannabinoids. Handb. Exp. Pharmacol. 168, 1–51.

Pertwee, R.G., 2009. Emerging strategies for exploiting cannabinoid receptor agonists as medicines. Br. J. Pharmacol. 156, 397–411.

Pertwee, R.G., Howlett, A.C., Abood, M.E., Alexander, S.P., Di Marzo, V., Elphick, M.R., Greasley, P.J., Hansen, H.S., Kunos, G., Mackie, K., Mechoulam, R., Ross, R.A., 2010. International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2. Pharmacol. Rev. 62, 588–631.

Pietr, M., Kozela, E., Levy, R., Rimmerman, N., Lin, Y.H., Stella, N., Vogel, Z., Juknat, A., 2009. Differential changes in GPR55 during microglial activation. FEBS Lett. 583, 2071–2076.

Prasad, A.S., 2008. Zinc in human health: effect of zinc on immune cells. Mol. Med. 14, 353–357.

Prasad, A.S., 2009. Zinc: role in immunity, oxidative stress and chronic inflammation. Curr. Opin. Clin. Nutr. Metab. Care 12, 646–652.

Rhee, M.H., Vogel, Z., Barg, J., Bayewitch, M., Levy, R., Hanus, L., Breuer, A., Mechoulam, R., 1997. Cannabinol derivatives: binding to cannabinoid receptors and inhibition of adenylylcyclase. J. Med. Chem. 40, 3228–3233.

Rieder, S.A., Chauhan, A., Singh, U., Nagarkatti, M., Nagarkatti, P., 2010. Cannabinoid-induced apoptosis in immune cells as a pathway to immunosuppression. Immunobiology 215, 598–605.

Rimmerman, N., Juknat, A., Kozela, E., Levy, R., Bradshaw, H.B., Vogel, Z., 2011. The non-psychoactive plant cannabinoid, cannabidiol, affects cholesterol metabolism-related genes in microglial cells. Cell. Mol. Neurobiol. 31, 921–930.

Rosenberg, G.A., 2009. Matrix metalloproteinases and their multiple roles in neurodegenerative diseases. Lancet Neurol. 8, 205–216.

Scuderi, C., Filippis, D.D., Iuvone, T., Blasio, A., Steardo, A., Esposito, G., 2009. Cannabidiol in medicine: a review of its therapeutic potential in CNS disorders. Phytother. Res. 23, 597–602.

Sensi, S.L., Paoletti, P., Bush, A.I., Sekler, I., 2009. Zinc in the physiology and pathology of the CNS. Nat. Rev. Neurosci. 10, 780–791.

Song, J., Lee, S.C., Kim, S.S., Koh, H.J., Kwon, O.W., Kang, J.J., Kim, E.K., Shin, S.H., Lee, J.H., 2004. Zn^{2+}-induced cell death is mediated by the induction of intracellular ROS in ARPE-19 cells. Curr. Eye Res. 28, 195–201.

Srivastava, M.D., Srivastava, B.I., Brouhard, B., 1998. Δ^9-Tetrahydrocannabinol and cannabidiol alter cytokine production by human immune cells. Immunopharmacology 40, 179–185.

Stella, N., 2010. Cannabinoid and cannabinoid-like receptors in microglia, astrocytes and astrocytomas. Glia 58, 1017–1030.

Streit, W.J., 2005. Microglia and neuroprotection: implications for Alzheimer's disease. Brain Res. Brain Res. Rev. 48, 234–239.

van der Stelt, M., Di Marzo, V., 2005. Cannabinoid receptors and their role in neuroprotection. Neuromol. Med. 7, 37–50.

Van Lint, P., Libert, C., 2007. Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation. J. Leukoc. Biol. 82, 1375–1381.

Walter, L., Stella, N., 2004. Cannabinoids and neuroinflammation. Br. J. Pharmacol. 141, 775–785.

Wimmer, U., Wang, Y., Georgiev, O., Schaffner, W., 2005. Two major branches of anti-cadmium defense in the mouse: MTF-1/metallothioneins and glutathione. Nucleic Acids Res. 33, 5715–5727.

Woelkart, K., Salo-Ahen, O.M., Bauer, R., 2008. CB receptor ligands from plants. Curr. Top. Med. Chem. 8, 173–186.