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Cannabidiol affects circadian clock core complex and its regulation in microglia cells

Geneviève Lafaye, MD[1,2], Christophe Desterke, PhD[3], Laurent Marulaz, MD[1,2] & Amine Benyamina, MD, PhD[1,2]

Dpt Addictologie, AP-HP, GH Paris-Sud, Hôpital Paul Brousse, Villejuif, France[1], INSERM U1178, Villejuif, France[2] and UFR Medecine, INSERM UMS 33, University Paris Saclay, Villejuif, France[3]

ABSTRACT

Cannabis is often used by consumers for sleep disorders. Studies show that circadian rhythm could be affected by a misuse of cannabis. Recent research has connected the role of microglial cells with psychiatric disorders such as substance abuse. The aim was to show the effect of two major components of cannabis on circadian genes regulation in microglial cells. In BV-2 microglial cells, cannabidiol (CBD) induces a deregulation of circadian genes with (*P*-value = 0.039) or without (*P*-value = 0.0015) lipopolisaccharides stimulation. CBD up regulated Arntl (P = 9.72E-5) and down regulated Clock (P = 0.0034) in BV-2 cells. Temporal expression of Arntl (light and dark P = 0.0054) and Clock (light and dark P = 0.047) was confirmed to have 24 hours light and dark rhythmic regulation in dissected suprachiasmatic nucleus as well as of Cb1 cannabinoid receptor (light and dark P = 0.019). In BV-2 microglia cells, CBD also up regulated CRY2 (P = 0.0473) and PER1 (P = 0.0131). Other nuclear molecules show a deregulation of circadian rhythm in microglial cells by CBD, such as RORA, RevErbα, RORB, CREBBP, AFT4, AFT5 and NFIL3. Our study suggests that circadian rhythm in microglial cells is deregulated by CBD but not by THC. It is consistent with clinical observations of the use of therapeutic cannabis to treat insomnia.

Keywords cannabidiol, circadian genes, microglial cells, THC.

Correspondence to: Geneviève Lafaye, Dpt Addictologie, Hôpital Paul Brousse, F94800 Villejuif, France. E-mail: genevieve.lafaye@aphp.fr

INTRODUCTION

All life forms have adapted their physiology taking in account the rotation of the planet on its axis (Thaiss *et al.* 2015). In humans, many physiological functions are regulated by the circadian rhythm, a cellular mechanism well understood today. Nycthemeral rhythm perturbations, whether of environmental or molecular causes, can have major consequences for health and especially for the development of addictions (Logan, Williams, & McClung 2014). For example, in humans, it has been reported that night work or 'jetlag' is associated with a higher rate of tobacco and alcohol use (Trinkoff & Storr 1998; Rogers & Reilly 2002). Inversely, addictions can create long-term sleep disturbances. Once an individual begins to experience alcohol abuse or other, this exposure produces changes in circadian rhythm and sleep in an acute and long-term manner, creating a vicious circle for those who already have abnormal circadian rhythm (Shibley, Malcolm, & Veatch 2008). These changes persist after discontinuation of the substance, which may be a factor contributing to relapse. The 24-hour biological rhythmicity is determined by the genes of the clock: these genes are independently expressed in the different cell types of the human body, but the main organ that coordinates this rhythmicity is the hypothalamus and, in particular, the suprachiasmatic nucleus (Welsh, Takahashi, & Kay 2010). BMAL1 protein is heterodimerized with the CLOCK or NPAS2 protein, which activates the translation of the PER and CRY genes whose mRNA accumulates in the nucleus of the cell during the morning. In the afternoon, the PER and CRY proteins are heterodimerized in the cytoplasm and then phosphorylated by Casein Kinase 1, which inhibits the activity of the BMAL1-CLOCK heterodimer during the evening (Takahashi 2015). Among the psychoactive substances found in addictions, cannabis is the most widely used illicit drug in France (Dujourdy & Besacier 2017) and also in the world (Niesink *et al.* 2015). Cannabis is constituted of several hundred different molecules, including 100 of cannabinoids (Dujourdy & Besacier

2017). The main psychoactive constituent is the delta-9-tetrahydrocannabinol (THC), and the second main cannabinoid is the cannabidiol (CBD). This last one has no hallucinatory effect and is rather considered, as the 'non-psychoactive' component (Babson, Sottile, & Morabito 2017). To characterize the strength of cannabis, the THC/CBD ratio is studied. Recent epidemiological studies performed in France revealed an increase in this ratio, so more THC as compared with CBD in illegal cannabis: ratio of 2 in 2009 and ratio of 6 in 2016 (Dujourdy & Besacier 2017). Therapeutic cannabis exists, and one of the most studied drugs is Sativex and has a ratio of 1/1. Cannabis is mainly used today because subjectively, it facilitates falling asleep (Conroy & Arnedt 2014). Objectively, in most polysomnography studies, THC has no effect on sleep time. On the contrary, chronically administered THC induces an effect of habituation of the hypnotic qualities, a weakening of the circadian rhythm, a daytime sleepiness and an increase of the time of falling asleep (in particular with high and prolonged doses in time) (Perron, Tyson, & Sutherland 2001; Nicholson *et al.* 2004; Vaughn *et al.* 2010; Gorelick *et al.* 2013). CBD has a waking effect at low doses and rather sedative at high doses (Zuardi 2008; Murillo-Rodríguez *et al.* 2014). The THC/CBD combination (Sativex) studied in the context of chronic pain has shown effects on the quality of sleep and on quality of life (Russo, Guy, & Robson 2007). A retrospective study of an online survey based on the testimony of patients using medical cannabis showed a decrease in the use of anxiolytics in 71.8 percent of patients, as well as a decrease in medications for sleep in 65.2 percent of patients (Piper *et al.* 2017). There is no link between clock genes and cannabis addiction. But there are genes whose expression depends on circadian genes: the 'clock-controlled genes'. And among them, the DRD2 gene, a dopaminergic D2 receptor gene, could be involved in cannabis addiction by its cross-expression with the cannabinoid type 1 (CB1) receptor gene, according to a study conducted in 2016, on a Turkish population (Isir, Baransel, & Nacak 2016). Also, through combined and innovative approaches at the cellular, molecular and genetic levels, links between glial cells, circadian rhythm cell function and sleep have recently been identified. It is known that glia–neuron communication in mice and Drosophila has a role in the circadian modulation of behaviors (Jackson *et al.* 2015). Also, exposure to drugs can affect microglial cells including those located in the brain regions involved in addictions: nucleus accumbens (Scofield *et al.* 2016), prefrontal cortex, ventral tegmental area, amygdala and hippocampus (Lacagnina, Rivera, & Bilbo 2017). Moreover, it is known that the endocannabinoid system may be sensitive to changes in the physiology of the microglial cell (Lisboa *et al.* 2016). Indeed, during microglial activation, by a stress or an inflammatory state, the CB2 receptors are more expressed (Walter *et al.* 2003). Also, microglial cells produce endocannabinoids at higher levels than neurons in vitro, suggesting that endocannabinoid production by activated glial cells may play an important role in neuroinflammatory processes. The endocannabinoids anandamide, palmitoylethanolamide and 2-arachidonoylglycerol affect immune function through CB2 receptors (Cabral, Rogers, & Lichtman 2015). The consequence of chronic cannabis use could implicate an abnormal glia–neuron communication. Endocannabinoid 2-arachidonoylglycerol was found to have a rhythmic circulating level, which increased continually across the morning and peaking in the early to midafternoon (Hanlon *et al.* 2015). Also, cannabinoid receptor signaling and especially via CB1 could control of feeding behavior at central level, which is daily regulated (Koch 2017). Ana Juknat (Juknat *et al.* 2013) has performed transcriptome study for the effect of CBD and THC treatments on B-V2 murine microglial cells. In the present work, we proposed to analyze effect of CBD and THC on circadian clock genes in BV2 glial cells.

MATERIAL AND METHODS

Transcriptome dataset performed on microglia BV-2 cells

Microglial cells are the resident macrophage-like cells of the central nervous system. BV-2 microglial cell line was immortalized by retrovirus J2 and over-expression of v-myc and v-raf oncogenes (Blasi *et al.* 1990). Cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) containing 4.5 g/L glucose, supplemented with 5 percent fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) under a humidified 5 percent CO2 atmosphere at 37°C. Cells were pretreated for 2 hours with either THC or CBD (both at 10 μM) followed by addition of lipopolysaccharides (LPS) (100 ng/ml) for another 4 hours. Transcriptome analysis performed on BV-2 murine microglial cell exposed to components of cannabis (THC or CBD) with or without LPS inflammatory condition (Juknat *et al.* 2013) was used to performed analysis. These experiments were downloaded from Gene Expression Omnibus (GEO) website (https://www.ncbi.nlm.nih.gov/geo/): original normalized transcriptome matrix GSE70689 (quantile normalization) was merged by SQL query with its corresponding GEO platform GPL7868 using technology VUmc/Illumina Sentrix MouseRef8 v1.1 (Illumina).

Next generation sequencing RNAseq dataset performed on suprachiasmatic nucleus in a temporal experiment map (24 hours light/dark cycle)

Suprachiasmatic nucleus is considered as the brain's circadian clock (Gillette & Tischkau 1999). In GEO dataset

GSE72095 (Pembroke *et al.* 2015), pooled dissected tissue of the suprachiasmatic nucleus from five adult male mice provided one of three replicates for each of six time points over a 12:12 light/dark cycle (ZT2, 6, 10, 14, 18 and 22) were treated. Each biological replicate was sequenced over three separate lanes using Illumina HiSeq 2000. Sequencing was performed with a 100-BP paired-end Illumina molecular library. Mapping between genome version mm10/GRCm38.70 and transcriptome was performed with the two algorithms: GMAP and GSNAP. Count tables were generated using HTseq software and normalized using DESeq2. Normalized count matrix was annotated with *Mus musculus* official gene symbol with Ensembl/Biomart database version 91. Light and dark temporal expression graph was performed with ggplot graphical definition in R-software version 3.4.1, and also, light and dark *P*-value was estimated by computing Kruskall–Wallis ranking test in the same environment software. The rhythmic expression of either clock-gene expression or cannabinoid receptors expression has been also processed in waveform analysis with R packages JTK_CYCLE, Bonferroni corrected (Hughes, Hogenesch, & Kornacker 2010) and with DeSeq2 algorithm with Benjamini–Hochberg correction for multitesting through SCNseq online software (http://www.wgpembroke.com/shiny/SCNseq/). Light and night fluctuation interpretation was accepted for a Deseq2 adjust P-value < 0.05 and JTK adjust P-value < 0.1.

Circadian core gene selection

In order to focused transcriptome analysis on circadian rhythm cell function, we query core circadian genes from specific literature (Takahashi 2015): Clock (clock circadian regulator), Per1 (Period Circadian Clock 1), Per2 (Period Circadian Clock 2), Per3 (Period Circadian Clock 3), Cry1 (Cryptochrome Circadian Clock 1), Cry2 (Cryptochrome Circadian Clock 2), Npas2 (neuronal PAS domain protein 2), Csnkle (casein kinase 1 epsilon), Csnk1a1 (casein kinase 1 alpha 1), Arntl (aryl hydrocarbon receptor nuclear translocator-like, alias Bmal1) and REV-ERB/ROR feedback loop genes: Nr1d1 (nuclear receptor subfamily 1 group D member 1, alias Rev-ErbA-Alpha), Rora (RAR-related orphan receptor A), Rorb (RAR-related orphan receptor B) and Rorc (RAR-related orphan receptor C).

Microarray analysis

Bioinformatics analysis on normalized microarray matrix was performed in R software environment version 3.0.2 'Frisbee Sailing'. Unsupervised principal component analysis was performed with FactoMineR R-package (Lê, Josse, & Husson 2008). Kaiser–Meyer–Olkin factor adequacy and Bartlett sphericity test were performed with 'psych' R package on correlation matrices of data processed in principal component analysis. Transcriptome heatmap was performed with function heatplot from made4 R bioconductor package (Culhane *et al.* 2005). Microarray gene set enrichment analysis (GSEA) was performed with GSEA standalone software version 2.2.0 (Subramanian *et al.* 2005) implemented on Msig database version 5.2. Functional enrichment on Gene ontology biological process for microarray analysis was performed with the website tools of Enrichr (Kuleshov *et al.* 2016). Network analysis representation realized on functional enrichment results was investigated in Cytoscape standalone software version 3.2.1 64bit (Cline *et al.* 2007).

Statistical analysis

Statistical analysis was performed also in R software environment version 3.0.2. Fisher's one-way ANOVA based on general linear model was performed for multi-comparison, unpaired mean comparison between two independent groups were performed with two-sided unpaired Student's t-test. For each test comparison, significance was assessed by rejection of null hypothesis for alpha inferior to 0.05.

RESULTS

1/Arntl/Bmal1 and Clock circadian core genes are regulated by cannabidiol in microglial cell independently of the inflammatory context

As cannabis is implicated in sleep regulation, clock gene regulation analysis was investigated in transcriptome dataset from murine BV-2 microglial cells treated or not with cannabis components: THC and CBD. These experiments were performed also in a context or not of inflammation (LPS stimulation). As BV-2 is an immortalized cell over-expressing v-raf and v-myc oncogenes, expression of raf and myc was checked in transcriptome data: Raf was not found in the matrix, and expression of Myc was not affected by CBD or THC stimulation as compared with control (data not shown). Also, it was checked if in vitro treatment performed on these cells could affect their phenotype by comparing the expression of Cd68 macrophagic differentiation marker, which was found highly expressed in BV-2 transcriptome (Fig. S1). Expression of Cd68 was not found regulated between each experimental condition (one-way ANOVA, P-value = 0.785). Firstly, core clock genes found in literature were investigated by unsupervised principal component analysis on their transcriptome expression: experimental group discrimination was found on first principal component axis concerning the LPS treatment.

On the second principal axis, group separation concerned principally the effect of CBD treatment in the context of inflammation (with LPS *P*-value = 0.0015) or not (without LPS *P*-value = 0.039), THC seems to have no effect on expression of core clock genes in these cells (Fig. 1a). This CBD effect was confirmed by unsupervised classification (Euclidean distances) on the sub-matrix concerning samples without LPS treatment (Fig. 1b) and on the sub-matrix concerning samples with LPS treatment (Fig. 1c). Boxplot of expression performed on Arntl (alias Bmal1) quantification revealed important up regulation of this transcription factor in the samples stimulated by CBD (Fisher's ANOVA *P*-value = 0.0026, Fig. 1d). Boxplot of expression performed on Clock quantification revealed down regulation of this transcription factor in the samples stimulated by CBD (Fisher's ANOVA *P*-value = 0.032, Fig. 1e). These results suggest that cannabidiol alias CBD has a significant effect on regulation of core clock genes in BV-2 microglial cells and especially on Bmal1 and Clock and these regulations were found independent of inflammatory context. In other way, THC seems to have no effect in these experiments.

2/Temporal expression of Cb1 cannabinoid receptor in suprachiasmatic nucleus follow a circadian light/dark rhythm like Arntl and Clock genes

Suprachiasmatic nucleus is considered as the brain's circadian clock (Gillette & Tischkau 1999). Next Generation Sequencing by RNAseq have investigated (GSE72095) one pool of five mice primary tissue suprachiasmatic nucleus with temporal experimental map exploring six time points over a 12:12 light/dark cycle (ZT2, 6, 10, 14, 18 and 22) on a cycle of 24 hours. Each experimental point is the pool of five males dissected and passed in triplicate for the RNAseq experiments. In these normalized expression data, we explored the temporal expression of cannabinoid receptors: Cnr1 (alias Cb1), Cnr2 (alias Cb2) and orphan receptors [Gpr18 (G protein-coupled receptor 18), Gpr55 (G protein-coupled receptor 55) and Gpr119 (G protein-coupled receptor 119)] (Irving *et al.* 2017) in parallel of circadian core genes regulated by CBD: Arntl/Bmal1 and Clock. Arntl and Clock circadian genes were confirmed to have an important expression and to have a light and dark rhythmic expression in suprachiasmatic nucleus (Fig. 2a) with a respective light and dark *P*-value of 0.0054 and 0.047. Arntl was confirmed to have waveform rhythmic expression during periodicity of the experiment (JTK *P*-adjust = 0.015, Fig. 2a). The expression of these two genes increased during dark period as compared with light period. Concerning the expression of cannabinoid receptors, only Cnr1 (alias Cb1 receptor) had an expression intensity level similar to the clock genes; Cnr2 (alias Cb2 cannabinoid receptor) and orphan receptor Gpr55 had a low expression level in suprachiasmatic nucleus (Fig. 2b). Orphan receptors Gpr18 and Gpr119 were found nearly undetectable in suprachiasmatic nucleus (Fig. 2b). Among Cannabinoid receptors, only Cnr1 receptor (Cb1) was found with a significant light and dark *P*-value (*P*-value = 0.019, Fig. 2b), with a double increase of expression during dark period as compared with light period, similar result found with Arntl and Clock genes (Fig. 2a). Cnr1 was confirmed to have waveform rhythmic expression during periodicity of the experiment (JTK *P*-adjust = 0.066, Fig. 2a). These results confirmed temporal expression of circadian genes Arntl and Clock in suprachiasmatic nucleus with an increase expression during night period and highlighted similar regulation for the cannabinoid receptor Cb1.

3/Expression of genes implicated in Circadian rhythm and its regulation mechanisms is altered in microglial cells traited by cannabidiol

Gene set enrichment analysis performed on transcriptome dataset of BHV2 in basal condition traited or not by CBD was investigated with MSigDB version 5.2. GSEA analysis performed with Gene Ontology-Biological Process gene set collection highlighted enrichment in CBD condition of functionalities connected to circadian rhythm such as Gene Ontology-Circadian Rhythm [normalized enrichment score (NES) = +1.42, *P*-value < 0.0001, Fig. 3a and Table S1]. GSEA analysis performed with Reactome Process gene set collection highlighted enrichment in CBD condition of functionalities connected to circadian rhythm such as Bmal1-Clock-Npas2 activates circadian expression (NES = +1.29, *P*-value < 0.0001, Fig. 3a and Table S1) and Circadian repression by RevErbA-alpha (NES = +1.34, *P*-value < 0.0001, Fig. 3a and Table S1). GSEA performed with gene ontology database allowed also to highlight a repression of circadian rhythm regulation (NES = −1.18, *P*-value < 0.0001, Fig. 3b and Table S1). Expression association of genes enriched in these different gene set allowed to perform a CBD sample group discrimination by unsupervised classification (Fig. 3c), suggesting that CBD has an important effect on regulation of all these circadian-related genes. Among best up regulated genes (Table S2), different transcription factors can be observed such as Atf4 (activating transcription factor 4), Atf5 (activating transcription factor 5), Nfil3 (nuclear factor, interleukin 3 regulated) and Arntl alias Bmal1 one of circadian core genes. Nuclear receptors such as Rora and Rxra were also found up regulated by CBD. Ppargc1a (PPARG coactivator 1 alpha) was also found slidely up regulated. Among downregulated genes, nocturnin was the most downregulated gene by CBD. It is interesting to notify downregulation of Nr1d1 (nuclear

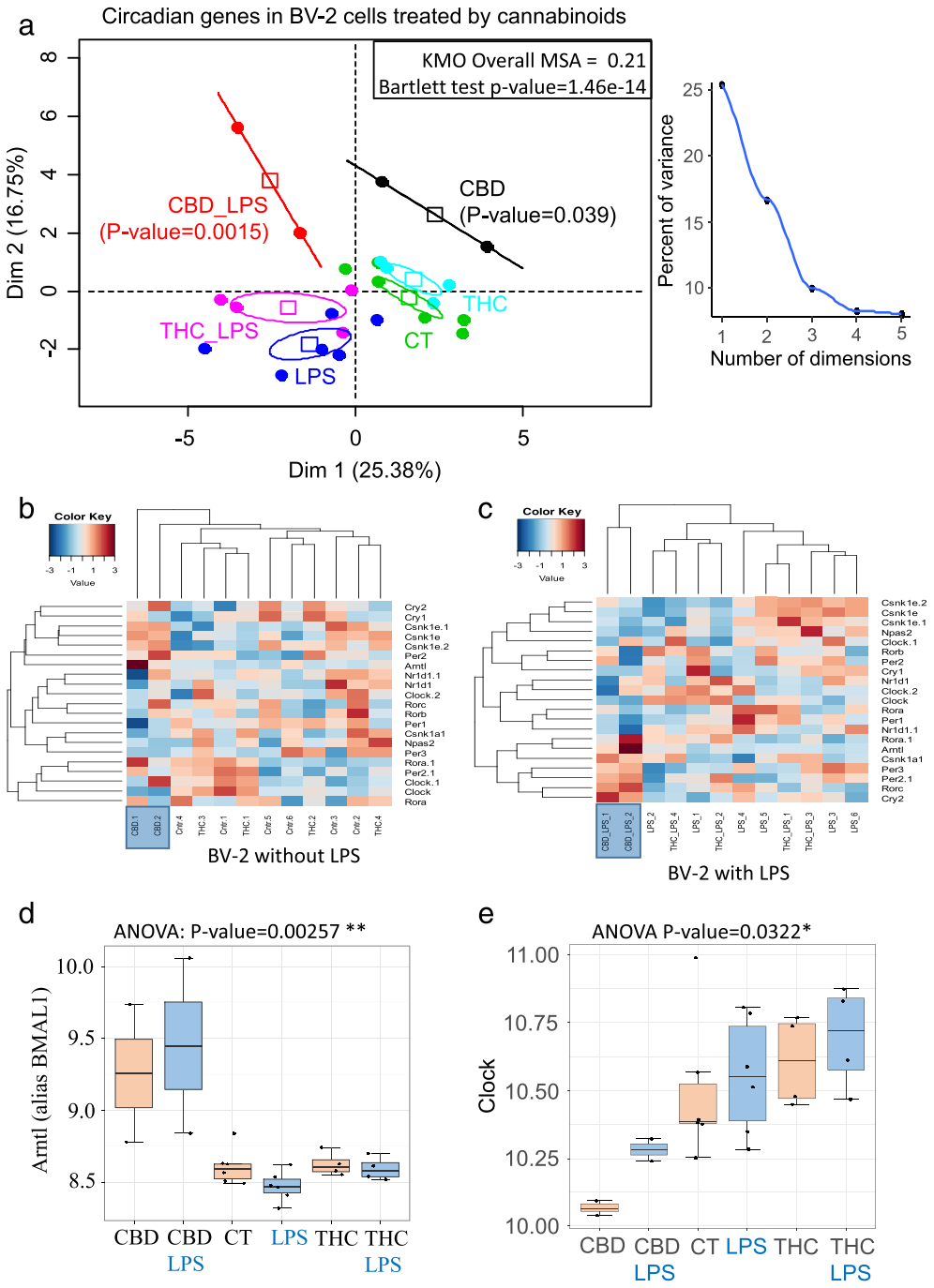

Figure 1 Bmal1 circadian core gene is regulated by cannabidiol (CBD) in microglial cells independently of the inflammatory context: (a) unsupervised principal component analysis performed with expression of circadian core gene quantification by microarray analysis performed on murine BV-2 microglial cells (GSE70689) stimulated with principal component of the cannabis [Δ9-tetrahydrocannabinol (THC) or CBD] with or without lipopolysaccharide (LPS) inflammatory condition (*P*-value was calculated with group separation correlated to the first principal axis), KMO index (Kaiser–Meyer–Olkin factor adequacy) and Bartlett sphericity test quality parameters results on the matrix were put as legend of the factorial map, and also, scatterplot for percent of variance on the five first axis of the principal component analysis is draw on the right of the factorial map; (b) unsupervised classification performed on circadian core gene expression sub-matrix of BV-2 cells without LPS treatment (GSE70689) (clustering was calculated on Euclidean distances), and blue sample cluster indicated discrimination of CBD samples; (c) unsupervised classification performed on circadian core gene expression sub-matrix of BV-2 cells with LPS treatment (GSE70689) (clustering was calculated on Euclidean distances), and blue sample cluster indicated discrimination of CBD samples; (d) boxplot of Arntl/Bmal1 microarray expression in murine BV-2 microglial cells (GSE70689) stimulated with principal component of the cannabis (THC or CBD) with or without LPS inflammatory condition (*P*-value of general regulation was evaluated by Fisher's one-way ANOVA); (e) boxplot of Clock microarray expression in murine BV-2 microglial cells (GSE70689) stimulated with principal component of the cannabis (THC or CBD) with or without LPS inflammatory condition (*P*-value of general regulation was evaluated by Fisher's one-way ANOVA)

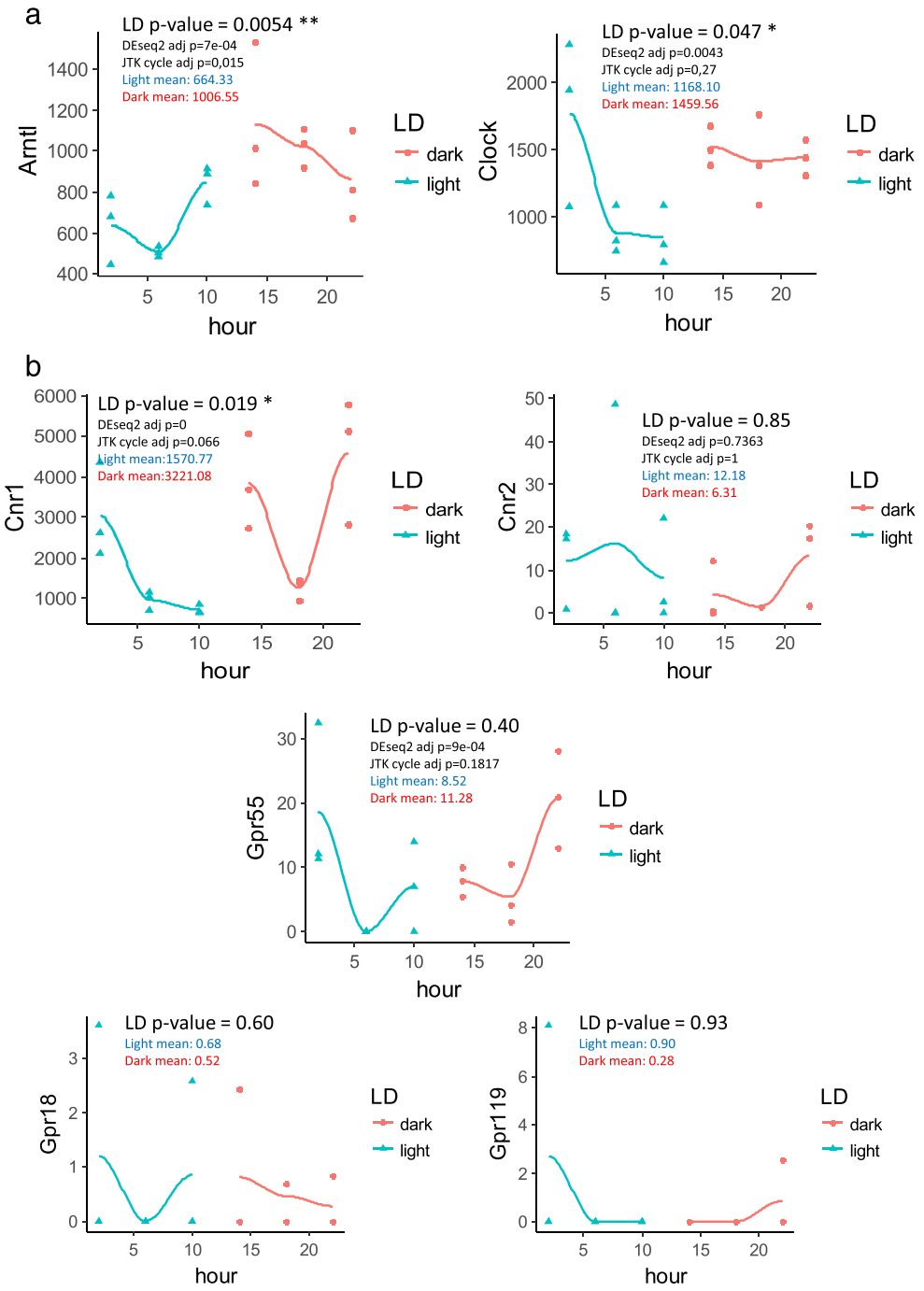

Figure 2 Temporal expression in suprachiasmatic nucleus for circadian genes regulated by cannabidiol and also for cannabiodiol receptors: (a) temporal expression graph on six time points over a 12:12 light/dark (LD) cycle in suprachiasmatic nucleus for Amtl and Clock genes: light and dark Kruskall–Wallis was indicated on the graph as well as expression means during light and dark periods; and (b) temporal expression graph on six time points over a 12:12 LD cycle in suprachiasmatic nucleus for Cm1, Cm2, Gpr55, Gpr18 and Gpr119 cannabinoid receptors: light and dark Kruskall–Wallis was indicated on the graph as well as expression means during light and dark periods; also, JTK_cycle adjust *P*-values and adjust *P*-values of Deseq2 algorithms were annotated

receptor subfamily 1 group D member 1) alias RevErbA-alpha, which is compatible with the previous gene set enrichment found in CBD condition: Reactome circadian repressed by RevErbA-alpha (Fig. 3a). It is interesting to notify also the downregulation of Mtmr1b (myotubularin-related protein 1) by CBD, which is the receptor 1B of the melatonin. Some dopamine receptors were also found downregulated by CBD: Drd2 (dopamine receptor D2) and less Drd4 (dopamine receptor D4). Corticotropin-releasing hormone (CRH) was also found slightly downregulated by CBD stimulation (Table S2 and Fig. 3c).

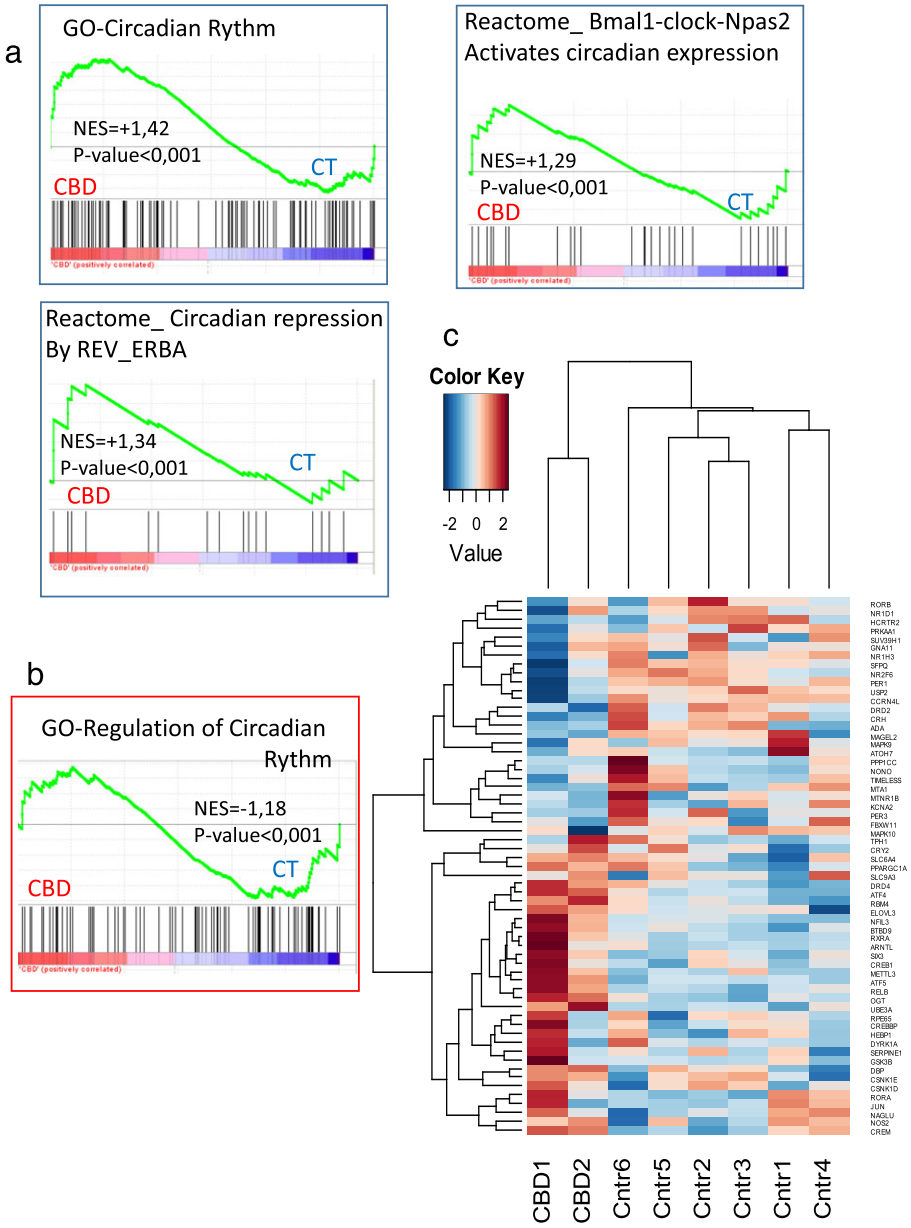

Figure 3 Genes implicated in Circadian rhythm and its regulation mechanisms are altered in microglial cells treated with cannabidiol: (a) gene set enrichment analysis of circadian rhythm functionalities performed with cannabidiol transcriptome dataset of BV-2 microglial cell in basal condition without inflammatory context (GO, gene ontology biological process database and Reactome database; NES, normalized enrichment score); (b) gene set enrichment analysis of circadian rhythm regulation performed with cannabidiol transcriptome dataset of BV-2 microglial cell in basal condition without inflammatory context; and (c) expression heatmap performed with unsupervised classification and with genes regulated by cannabidiol in BV-2 microglial cell (Cntr, control unstimulated cells; CBD, cannabidiol stimulated cells)

4/Circadian clock-related genes affected by cannabidiol in microglial cells implicated melatonin metabolism

Functional enrichment performed with circadian rhythm-related genes on wikipathway database version 2016 revealed important implication of melatonin metabolism and its effect (Fig. 4a) principally by regulation of core circadian genes such as per1 (Period Circadian Clock 1), Per3 (Period Circadian Clock 3), Cry2 (Cryptochrome Circadian Clock 2) and Arntl (Fig. 4b). During gene set enrichment analysis highlighted a downregulation of Mtnr1b by CBD treatment, which is coding for a melatonin receptor (Fig. 3b and Table S2). Melatonin metabolism, as several other functions, was found connected to Gsk3b, which seems to play central role by its high connectivity in the network (Fig. 4b). Sudden Infant Death Syndrome Susceptibility pathway was also found importantly represented during this functional enrichment especially by different molecules implicated in cyclic AMP response such as Crem-1

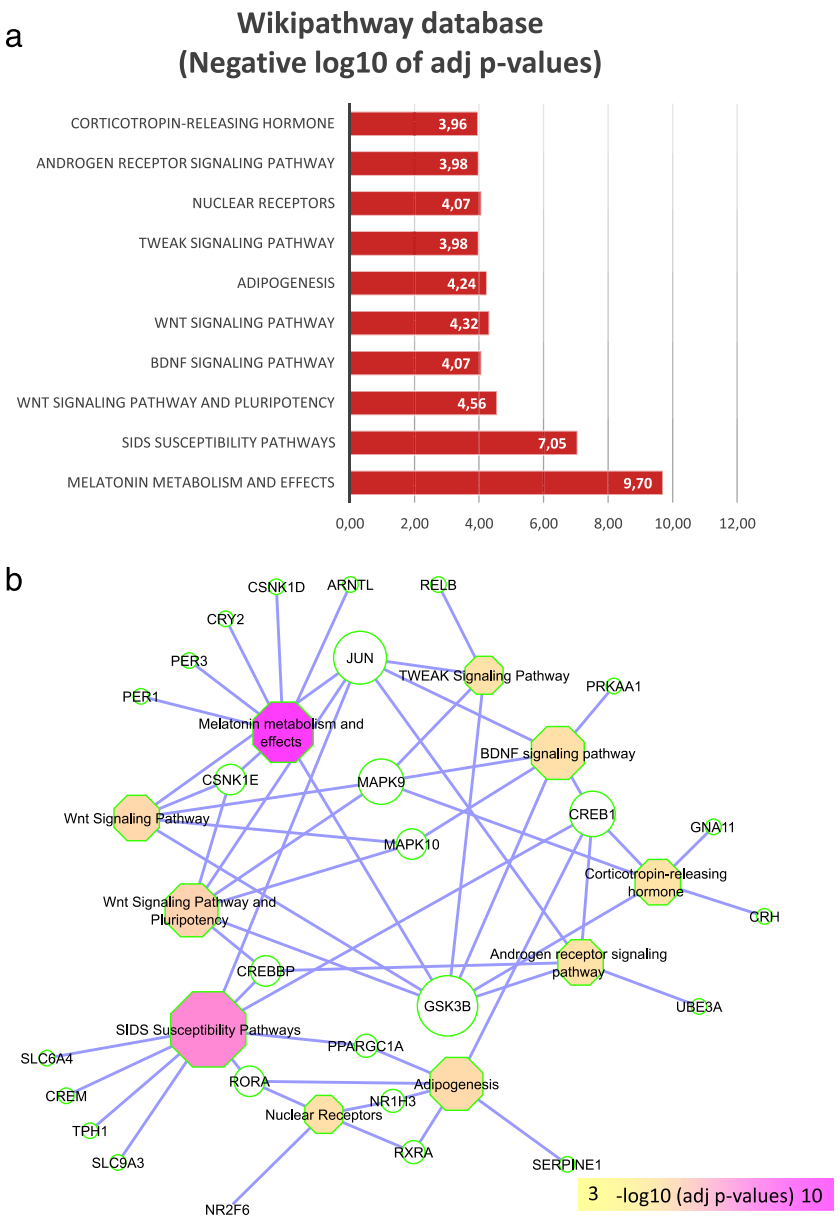

Figure 4 Circadian clock-related genes affected by cannabidiol in microglial cells implicated melatonin metabolism: (a) barplot of functional enrichment performed on wikipathway 2016 database with circadian clock-related genes regulated by cannabidiol in microglial cells (*x* axis: negative logarithm of base 10 from the adjust *P*-value obtained with enrichr webtools); and (b) functional enrichment network analysis performed with wikipathway enrichment analysis of clock-related genes regulated by cannabidiol in microglial cells: octagons represent enriched functions, circles represent enriched genes over expressed in HO, edges represent connections between genes and functions, fill color of the octagons are proportional to the negative log10 of enrichment *P*-value and size of the element is proportional to the number of direct connections to the elements

(Cyclic AMP response element modulator-1), Creb1 (CAMP responsive element-binding protein 1) and Crebbp (CREB-binding protein) (Fig. 4a and b) and the serotonin transporter Slc6a4 (solute carrier family 6 member 4).

5/Core circadian gene complex and genes implicated in circadian rhythm are affected by cannabidiol independently of the inflammatory context in microglia

Unsupervised principal component analysis performed with circadian rhythm-related genes on microglia transcriptome samples with or without treatment by CBD (cannabis component) and LPS (inflammatory context) revealed a significant discrimination on the first principal axis (*P*-value = 3.82E-5) between samples treated and untreated by CBD independently of the inflammatory context (Fig. 5a). Circadian rhythm-related genes found correlated to the first principal axis (Table S3) were used to perform unsupervised classification represented by expression heatmap (Fig. 5b): CBD treated samples independently of their LPS stimulation were found classified in a distinct cluster than untreated samples. Among these genes, some circadian core genes were found significantly up regulated in CBD samples as compared with unstimulated samples (Arntl: two-sided Student's *t*-test *P*-value = 9.72E-5, Cry2: two-sided Student's *t*-test *P*-value = 0.047, Fig. 5c), and some were found significantly downregulated in CBD samples as compared with unstimulated samples (Clock: two-sided Student's *t*-test

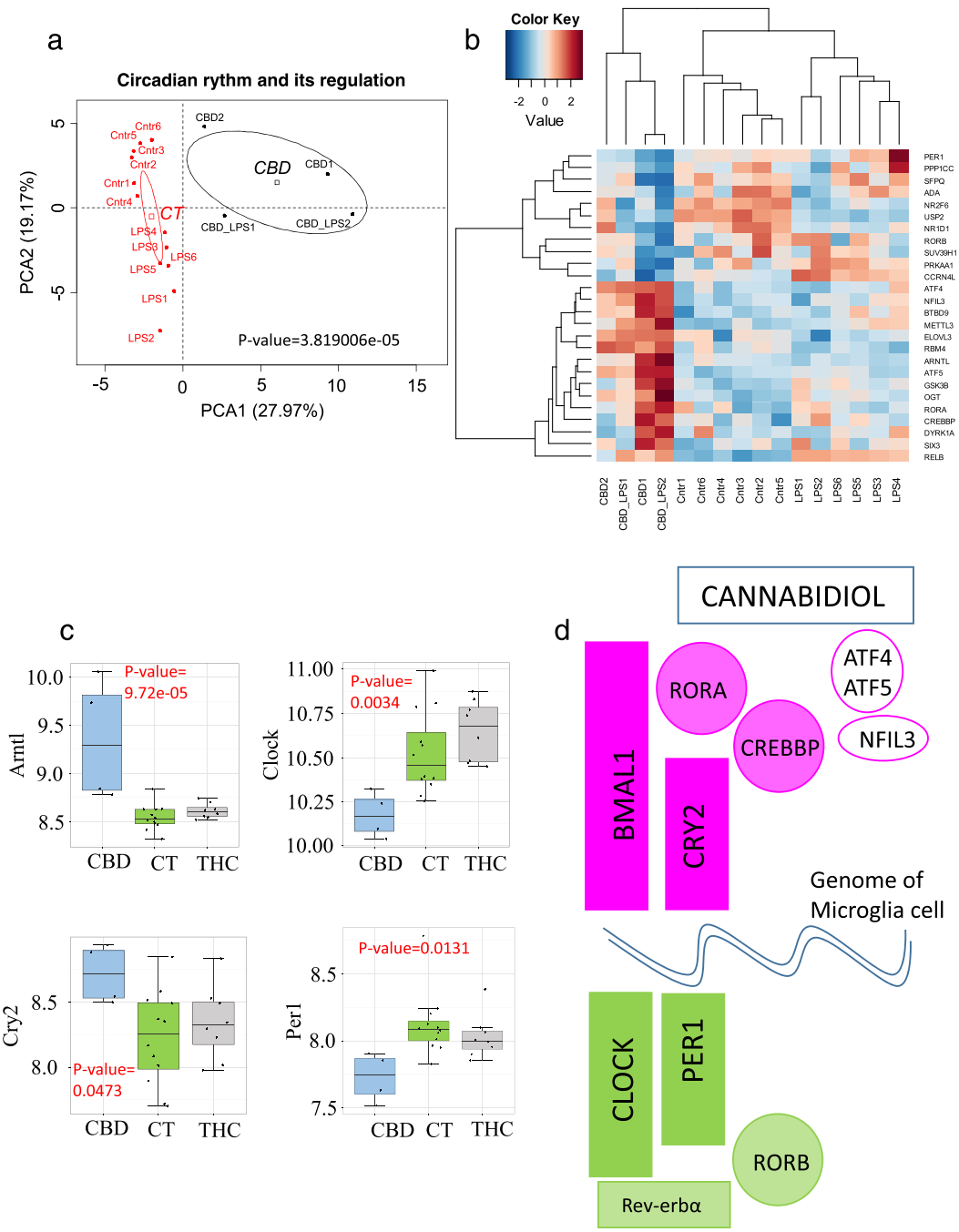

Figure 5 Genes implicated in Core circadian complex and circadian rhythm are affected by cannabidiol (CBD) independently of the inflammatory context in microglia: (a) unsupervised principal component analysis performed with genes implicated in circadian rhythm and its regulation (samples in red: control condition without CBD stimulation but with or without stimulation by LPS; samples in black: samples stimulated by CBD and with or without stimulation with LPS, and *P*-value was estimated by correlation of the variable group on the first principal component axis); KMO index (Kaiser–Meyer–Olkin factor adequacy) and Bartlett sphericity test quality parameters results on the matrix were put as legend of the factorial map, and also, scatterplot for percent of variance on the five first axis of the principal component analysis draws on the right of the factorial map; (b) expression heatmap performed with circadian-related genes correlated to the CBD stimulation in the dataset of BV-2 cells; (c) expression boxplot performed on core clock genes found significantly regulated by CBD in microglial cell independently of the inflammatory context (*P*-value was calculated by two-sided Student's *t*-test between control unstimulated condition and CBD treated condition); and (d) draw summarizing the core clock and circadian-related transcription factors deregulated by cannabidiol in microglial cells (red: up regulated; green: downregulated; fill bar: core clock gene; fill circle: circadian neighbors transcription factor; and empty circle: transcription factor co-regulated and implicated circadian rhythm and its regulation)

P-value = 0.0034, Per1: two-sided Student's *t*-test *P*-value = 0.013, Fig. 5c). In order to summarize transcription factors regulated by CBD in the current study, a draw was performed (Fig. 5d): among other circadian core genes, Nr1d1 alias RevErbAα tends to be downregulated. Some nuclear hormone receptors (Rora and Rorb) were found regulated, and also, Crebbp implicated in cyclic AMP response was found up regulated. Atf4, which regulates the circadian expression of the core clock component Per2, was found particularly up regulated by CBD (Fig. 5d and Table S2). Atf5, an important regulator of the cerebral cortex formation, implicated also in cyclic AMP response was also found up regulated (Fig. 5d and Table S2). Finally, nuclear factor Nfil3 that binds as homodimer ATF sites was found up regulated and is known to be implicated in circadian rhythm by repressing expression of Per1 and Per2 (Fig. 5d). All these results suggest major deregulation of circadian rhythm and its regulation during CBD stimulation of microglial cells.

DISCUSSION

Results interpretation

In theory, CBD seems to maintain the microglial cell (of BV-2 mice) in a state of early awakening, if we take in account expression of the clock genes; dimerization of BMAL1 leads to the expression and accumulation of PER proteins in the morning (Logan *et al.* 2014). Arntl/Bmal1 gene up regulation, combined with the negative regulation of the Per1 gene, among others, would block the natural cycle of the cell as it is in the morning. Furthermore, the negative feedback of Clock can be considered to have only a minor impact, given the possibility of transcriptional compensation by NPAS2 protein.

Also, the downregulation of the melatonin 1B receptor suggests that resting is prevented. These results suggest that CBD, by its significant effect on the expression of the main clock genes in BV-2 microglial cells, blocks cells in a waking state; also, this regulation was independent of the inflammatory context. On the other hand, THC seems to have no effect on these tests. This is consistent with the clinical observation of the CBD stimulatory effect administered at low doses (Murillo-Rodríguez *et al.* 2014).

Nevertheless, clinical studies also show a sedative effect of CBD at high doses (Nicholson *et al.* 2004; Zuardi 2008). This can be explained by the negative regulation of the CRH gene, which theoretically leads to a decrease in cortisol. However, in a normal circadian rhythm, there is a rise in the rate of cortisol in the morning, and it is known that corticosteroids can lead to an improvement in waking state, or even go to a manic state. It can therefore be deduced that a drop in CRH leading to a fall in cortisol can lead to a sedative effect. Moreover, the nocturnin gene action, which is negatively regulated by CBD, remains to be clarified. Also, during this work, it had been expected to see a difference between inflammatory and non-inflammatory conditions. Indeed, stress (potentially under stimulation by LPS during these experiments) is known to induce activation of cannabinoid CB2 receptors, which leads to production of endocannabinoids that affect immune function via CB2 (Walter *et al.* 2003; Cabral *et al.* 2015). This lack of difference between LPS and control conditions suggests that CBD could have a mechanism of action independent of the cannabinoid receptors and the neuroinflammatory state in this cell type.

Finally, we have shown temporal expression (light and night cycle) of Cnr1 coding for Cb1 receptor in central clock of suprachiasmatic nucleus. This endocannabinoid receptor as compared with Cb2 is the principal one known to be implicated in psychoactive effect of cannabis. According to the data obtained with CBD treatment, the cannabis through this component appears to cause sleep disturbance by its impact on circadian clock gene regulation. However, a higher expression of Cb1 receptors in the evening and at night could also explain that consumers are shifting. As the expression level of the receptors was found more important during night period, maybe for this reason, consumers use cannabis at that time to increase its psychoactive effects. There is no current literature data on a link between the circadian clock and genes coding for cannabinoid receptors. This discovery, combined with clinical data of cannabis users, therefore justifies the development of further studies.

Big points and limits

The murine model is a solid experimental model for approaching the functioning of the circadian rhythm in human, because this system is common to all mammals.

Immunopsychiatry has grown significantly in recent years, and cannabis may play a major role in this development. Indeed, microglial cells are cells of immunity at the cerebral level, and their link with psychiatric manifestations have already been established (Lisboa *et al.* 2016).

Nevertheless, there are certain limitations to this study, which are related to the retrospective scheme and the fact that the experiments were performed in vitro, which does not necessarily reflect the complex reality in vivo, and the various molecular interactions. Also, microglial cells are present in different regions of the brain, and it is difficult to extrapolate their mode of functioning as being identical in each brain region. Indeed, according to the experiments, we find molecular mechanisms in microglial cells that are specific to certain brain

regions: prefrontal cortex (Zamberletti *et al.* 2015) and cerebellum (Cutando *et al.* 2013).

Comparison of the data to the literature

When we compared our data with the international scientific literature, we realized that this is the first study specifically examining the effect of THC and CBD on clock gene expression. Nevertheless, some studies already existed on the role of clock genes in addictions (Forde & Kalsi 2017). Few articles have studied the effect of certain drugs on the expression of clock genes in different brain regions. In particular, in rodents, there is an alteration of the ventral tegalic area and mesolimbic regions, after acute or chronic administration of drugs (Webb *et al.* 2009). Acute administration of psychostimulants (cocaine and methamphetamine) altered the expression of PER1 and 3, CRY1, BMAL1, NPAS2 or CLOCK in the Accumbens nucleus, dorsal striatum or hippocampus (Nikaido *et al.* 2001; Iijima *et al.* 2002; Yuferov *et al.* 2003; Falcon *et al.* 2013). Chronic administration of cocaine has also influenced PER, CRY1, CLOCK, BMAL1 or NPAS2 gene expression in these cerebral regions (Yuferov *et al.* 2003; Lynch *et al.* 2008; Falcon *et al.* 2013; Ozburn *et al.* 2013). Nevertheless, the effect (i.e. increase or decrease) depends on the gene and brain region examined, the duration of administration and the sampling time. Also, regular daily dosing with methamphetamine can alter the striatal expression of PER1 and 2 (Iijima *et al.* 2002; Natsubori, Honma, & Honma 2013). In addition, daily administration of a dopaminergic agonist may increase PER2 expression in this cerebral region (Gallardo *et al.* 2014). Among the studies linking clock genes and addictions, a mutation in the CLOCK gene has been identified in mice (CLOCK-Δ19) (Kennaway *et al.* 2003), conferring a manic phenotype, hyperhedonic, with reduce of sleep and vulnerability to cocaine (Ozburn *et al.* 2012). However, it is known that substance use disorders, including alcohol, often have co-morbidity with bipolar disorder (Fossey *et al.* 2006).

Furthermore, in the blood cells of patients with alcohol dependence, as well as in individuals with social alcohol consumption, the expression of BMAL1 was found significantly reduced (Ando *et al.* 2010; Huang *et al.* 2010). Also, the literature showed a correlation between the rate of cortisol and relapse in alcohol-abstinent patients (Walter *et al.* 2006). However, our results show that CBD negatively regulated the CRH gene and positively the BMAL1 gene. This therefore provides a theoretical basis for setting up a therapeutic trial of CBD in hospitalized patients for alcohol withdrawal, in the prevention of relapse.

In conclusion, disorders related to cannabis use are particularly common in France and are often accompanied by sleep disorders. In addition, there is growing interest in microglial cells and their role in circadian rhythm cell function and addictions. It is known that cannabis, in all its forms, can have multiple effects on circadian rhythm cell function and microglial cells. Our study revealed a significant effect of CBD on the expression of clock genes in murine BV-2 microglial cells, unlike THC, and independently of the inflammatory context. These results are consistent with the international literature indicating a stimulating effect of CBD at low doses. Nevertheless, they contradict the 'non-psychoactive' presentation that is made of it. Indeed, CBD, by acting on cells of the central nervous system and resulting in an effect on behavior (waking state), could be called psychotropic in itself. Also, our results highlight the importance of continuing research in order to find the best indications of therapeutic cannabis. Medical cannabis is indeed a way to explore in the field of addictology, given the interaction between the cannabinoid system and clock genes. These investigations will require prospective, randomized, double-blind studies in targeted populations, with special attention to sleep (polysomnography) and attention to ratios (THC/CBD), doses, duration of treatment and tolerance.

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AUTHORS CONTRIBUTION

CD, GL, LM and AB assisted with data analysis and interpretation of findings. CD performed the transcriptome analysis. CD, GL, LM and AB drafted the manuscript. GL and CD provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Expression of Cd68 macrophagic differentiation marker in BV-2 microglia cells:Expression of Cd68 macrophagic differentiation marker across the different condition of stimulation of BV-2 with cannabinoids (CBD and THC) and Lipopolysaccharides. Variation of expression between groups was estimated by performing Fisher one way ANOVA.

Table S1. table of the gene sets enriched during gene set enrichment analysis between cannabidiol stimulated cells and control cells unstimulated: this table corresponds to each enriched gene set gene sets which were found up regulated with cannabidiol stimulation and the gene set of regulation found down regulated with cannabidiol stimulation. Gene set enrichment analysis parameters are provided: ranking position of the gene in the corresponding gene set, ranking position of the totality of the microarray and running enrichment score

Table S2. table of expression fold change for the circadian rhythm related genes regulated by cannabidiol in microglial cells: for each gene found regulated by cannabidiol (CBD) in microglial cells fold change was calculated between CBD microarray condition and basal condition unstimulated, rows in red represent genes up regulated by CBD and row in green represent genes down regulated by CBD

Table S3. table of circadian rhythm related genes found correlated to cannabidiol stimulation independently of

the inflammatory context in microglia cells: for each circadian rhythm related genes found correlated in their expression to the CBD stimulation in microglial cell and independently of LPS stimulation the R Pearson correlation coefficient and corresponding p-values are describe in the table, red rows correspond to genes with a positive correlation to the CBD stimulation and green rows correspond to genes with a negative correlation to the CBD stimulation