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Concentration-dependent effect of delta-9-tetrahydrocannabinol on epigenetic DNA modifiers in human peripheral blood mononuclear cells

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Cannabis is among the most used illicit substances in the world, and approximately 10% of regular cannabis users are estimated to be susceptible to developing cannabis use disorder (CUD). We examined the effect of different concentrations of delta-9-tetrahydrocannabinol (THC) on the epigenetic DNA modifiers DNA methyltransferases (DNMTs) and ten-eleven translocation enzymes (TETs); cannabinoid *CB1* and *CB2* receptors; and the cytokines *IL-1β, IL-6*, *IL-10*, and *TNF-α*. We used two in vitro study designs on human peripheral blood mononuclear cells (PBMCs) collected from healthy donors: (a) repeated THC incubations and (b) repeated THC incubations followed by an "abstinence" period and a THC challenge incubation. We observed no significant effects on *DNMTs* and *TETs* mRNA levels, enzymatic activity, or *CB1* and *CB2* mRNA levels at an average THC concentration (50 ng/ml, $n = 8$ donors). However, repeated incubations at a high THC concentration (200 ng/ml, $n = 16$ donors) significantly downregulated *DNMTs* and upregulated *TETs, CB1*, and *CB2* mRNA levels. Both THC concentrations upregulated the gene expression of *IL-1β, IL-6*, and *IL-10*, but had no effect on *TNF-α* gene expression. At the genome-wide level, repeated THC incubations resulted in a significant number of differentially hydroxymethylated genes being hyperhydroxymethylated. An additional THC challenge shifted the hyperhydroxymethylated state to hypohydroxymethylation. The genes with the strongest associations with THC exposure were found to be functionally significant for various signaling pathways. These findings suggest that repeated incubations with high concentrations of THC may affect the expression of genes critical for the development of CUD through aberrant demethylation.

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INTRODUCTION

Cannabis is one of the most used illicit psychoactive drugs globally. As its prevalence continues to rise, daily or near-daily use is becoming increasingly common, and approximately 10% of regular users are at risk of developing cannabis use disorder (CUD) [1–3].

The primary component in the cannabis plant responsible for the psychoactive and rewarding effects is delta-9-tetrahydrocannabinol (THC), which makes higher THC content in cannabis samples a risk factor for the development of CUD [4]. THC exerts its effects in the body by binding to cannabinoid receptor 1 (CB1), which is located primarily in the nervous system, and cannabinoid receptor 2 (CB2), which is found mostly in the cells and tissues of the immune system [5–7].

Epigenetic modifications have been investigated as potential mechanisms that could cause maladaptive synaptic plasticity in brain regions related to drug-seeking behavior. Thus contributing to complex processes of adaptation and memory formation, which play a role in causing long-lasting behavioral changes and, ultimately, the development of drug addiction [8–10]. One epigenetic modification of interest is DNA methylation, in which DNA methyltransferases (DNMT 1, 3 A, 3B) add a methyl group to the fifth position of cytosine, forming 5-methylcytosine (5-mC). Cytosine methylation most commonly occurs in the promoter regions of genes and is generally associated with transcriptional repression [11, 12]. Conversely, DNA demethylation leads to the removal of methyl groups through a multistep process mediated by ten-eleven translocation enzymes (TET 1–3). The first step in the process involves the addition of a hydroxyl group to 5-mC, resulting in the formation of 5-hydroxymethylcytosine (5-hmC) [13, 14].

The epigenetic effects of cannabis have not been thoroughly studied despite the overall increase in cannabis consumption [15]. Genome-wide association studies have identified significantly differentially methylated genes associated with cannabis usage [16–19]. Reduced genome-wide methylation has been reported in the peripheral blood of adolescent chronic cannabis users [20] and in the human granulosa cells of cannabis-using women [21]. Another recent study revealed an association between cannabis smoking and changes in the expression of DNMTs and cannabinoid receptors [22]. Altered *CB1* methylation in peripheral blood cells was also reported in one candidate gene association study [23].

Our research team developed and validated an in vitro model of human peripheral blood mononuclear cells (PBMCs) to examine the impact of addictive substances on DNA-methylating enzymes

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[24, 25]. In addition, it has been established that PBMCs express cannabinoid receptors, dopamine receptors, dopamine transporter, and tyrosine hydroxylase [26, 27]. An advantage of the PBMC model is that cells can be solely exposed to THC without any other confounding factors, increasing the reliability of the results of our study in assessing the effect of THC on DNA methylation in humans.

At present, it is unclear why occasional cannabis use in some individuals leads to CUD. In this study, we focused on THC-induced aberrant DNA methylation, which may have long-term effects on the expression of genes in PBMCs. Our aim was to clarify how different concentrations of THC (50 and 200 ng/ml) affect epigenetic DNA modifiers (DNMTs and TETs), CB1 and CB2, and cytokines at the gene expression level. According to our hypothesis, in addition to having a direct effect on the central nervous system, repeated exposure to a high concentration of THC (200 ng/ml) might also produce more substantial epigenetic and gene expression changes in PBMCs than THC at lower concentrations (50 ng/ml). These changes could enhance the ability of cytokines (e.g., interleukins) to penetrate the brain, thereby influencing neuroplasticity. THC-induced changes in PBMCs also provide information on possible dysfunctions of the peripheral immune system. This may be one underlying mechanism for CUD comorbidities, such as major depression, posttraumatic stress disorder, and chronic pain [28].

The THC concentrations 50 and 200 ng/ml chosen for our study should reflect an average and high THC concentration in the blood after cannabis smoking and are based on studies on cannabis users' serum THC levels [29–32]. In this study, we used PBMCs from healthy men, but we assume that similar changes can occur elsewhere in the body, including in the brain [24].

METHODS AND MATERIALS

Isolation of PBMCs from the whole blood of healthy donors

Fresh whole blood was collected from healthy male volunteers ($n = 16$) aged between 20 and 40 at the Tartu University Hospital Blood Bank. The volunteers confirmed that they had not used narcotics in the last 12 months. Human PBMCs were isolated using a Ficoll-Paque density gradient (GE Healthcare, USA) on the day after blood collection. PBMCs were stored at −150 °C until further use.

Study design and THC incubations

PBMCs were thawed 24 h before the incubations and cultured in RPMI-1640 medium (Gibco, Thermo Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, CA, USA) and 1% penicillin–streptomycin solution (Pen Strep; Gibco, Thermo Scientific, MA, USA) in 24-well plates. THC (Dronabinol – synthetic oral delta-9-tetrahydrocannabinol) was obtained from Lipomed (Lipomed AG, Switzerland) in 1 ml/ml ethanol.

Two different study designs were used:

1. **Repeated THC incubations (R-THC):** PBMCs were incubated with THC (50 ng/ml, $n = 8$ and 200 ng/ml, $n = 16$) or a corresponding amount of vehicle (ethanol solution) for 1 h on five consecutive days. PBMCs were collected 24 h after each incubation.
2. **Repeated THC incubations+THC challenge (THCch):** PBMCs were incubated with THC (200 ng/ml, $n = 16$) or vehicle (ethanol solution) for 1 h on five consecutive days, which were followed by five days of abstinence. After the abstinence, one additional incubation with THC (200 ng/ml) or vehicle was performed, and all the cells were collected 24 h after the last incubation.

The purpose of the **R-THC** study was to imitate daily cannabis use, and our second study design, **THCch** the pattern of recreational cannabis use.

PBMCs were supplemented with fresh medium after all R-THC and THCch incubations and on the third day of the abstinence period and were grown at 37 °C in an atmosphere of 95% air and 5% CO_2.

The collected cells were prepared into cell lysates, RNA, DNA, and nuclear extracts and stored at −80 °C until further use. A detailed visual representation of the study design is presented in Fig. 1, and additional clarifications can be found in the Supplementary Materials.

mRNA analysis by RT–qPCR

Total RNA was extracted from the PBMCs using a RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from 400 ng of total RNA using the First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). RT–qPCR was

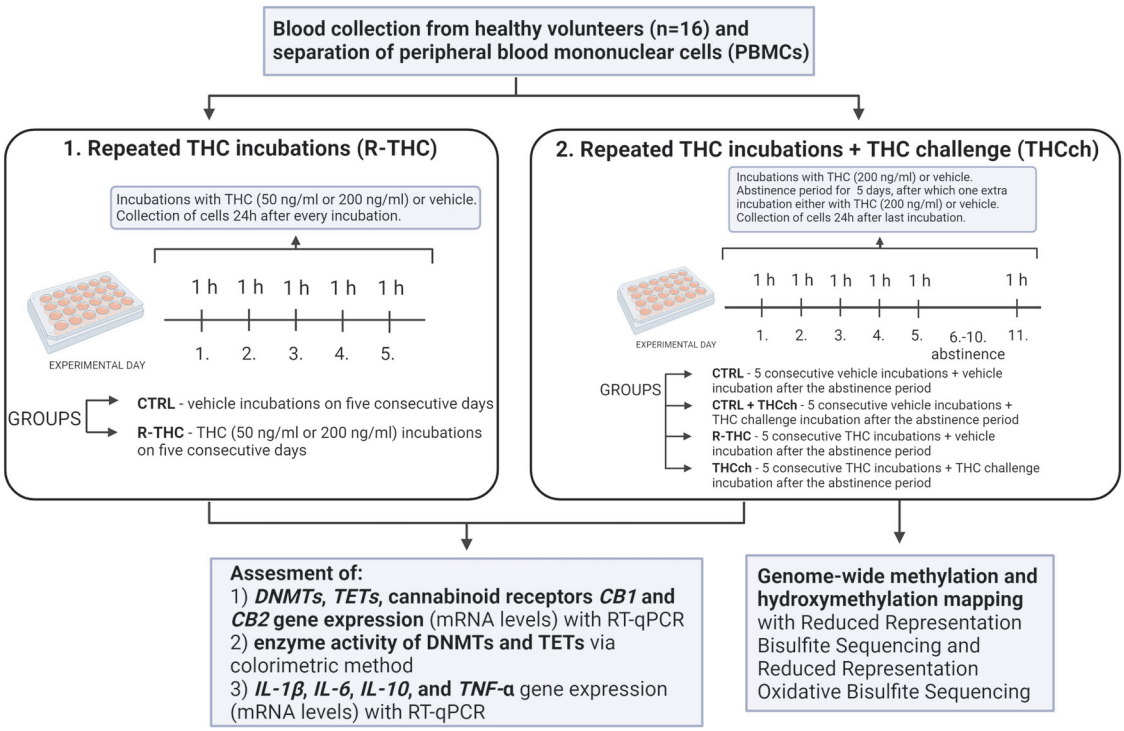


Fig. 1 Visual representation of the study design. Created with BioRender.com.

performed using QuantStudio 12 K Flex Software (Thermo Scientific, Waltham, MA, USA). The primers were designed using Primer3 via BLAST sequence verification and synthesized by TAG Copenhagen AS (Denmark). The list of primers used can be found in Supplementary Table 1. All the samples were amplified in duplicate, and the results were normalized to *B2M* using the comparative $C_T(2^{-\Delta\Delta CT})$ method [33].

DNMTs and TETs activity measurements

Nuclear proteins were extracted from the PBMCs according to the manufacturer's protocol (Nuclear Extraction Kit; ab113474; Abcam, Cambridge, UK). Following the manufacturer's instructions, DNMTs activity was measured using the Abcam DNMT activity assay kit (ab113467), and TETs activity was measured using the TET Hydroxylase Activity Quantification kit (ab156912).

Reduced representation bisulfite sequencing (RRBS) and reduced representation oxidative bisulfite sequencing (RRoxBS)

DNA from the PBMCs ($n = 8$) was extracted using the QIAamp DNA Mini Kit according to the manufacturer's protocol (QIAGEN, Hilden, Germany). The samples were sent to CD Genomics (The Genomics Services Company, USA) for library preparation, sequencing, and bioinformatics analysis. A detailed description of the RRBS and RRoxBS workflows and bioinformatics analyses can be found in the Supplementary Materials.

Statistical analysis and data visualization

Gene expression and enzyme activity data were analyzed using GraphPad Prism 10.1.1 software (San Diego, CA, USA). The normality of the qPCR and enzyme activity data sets was determined by Shapiro–Wilk's test and QQ plot evaluation (Supplementary Tables 2–6). mRNA levels and enzyme activity were analyzed with Student's t-test, one-way ANOVA with Tukey's multiple comparisons test, or two-way repeated measures ANOVA with Šidak's multiple comparisons test. The Mann–Whitney test with Holm–Šidak's multiple comparisons test or the Kruskal–Wallis test with Dunn's multiple comparisons test were used for non-normally distributed data. The significance level was set to $p < 0.05$. The detailed two-way repeated measures ANOVA statistical values are given in Supplementary Table 7. RRBS and RRoxBS data were visualized using the R package "ggplot2".

RESULTS

R-THC and THCch incubations alter *DNMTs* and *TETs* gene expression and activity

RT-qPCR results revealed that repeated incubations with THC at concentration 50 ng/ml did not affect *DNMTs* or *TETs* gene expression over the course of five days (Supplementary Fig. 1A). However, a THC concentration of 200 ng/ml had a statistically significant effect on *DNMTs* and *TETs* gene expression (Fig. 2A). Specifically, compared to the control group over five days, *DNMTs* mRNA levels decreased with the greatest changes observed 24 h after the third consecutive incubation (*DNMT1*, *DNMT3A*, *DNMT3B*, $p < 0.0001$; Holm–Šidak's post hoc test; $n = 16$). The opposite occurred for *TETs*, as their mRNA levels increased, with the greatest changes observed 24 h after the third incubation compared to the control group (*TET1*, *TET2*, $p < 0.0001$; Holm–Šidak's post hoc test; $n = 16$; *TET3*, $p < 0.0001$; Šidak's post hoc test; $n = 16$). However, a subsequent decrease followed, and both *DNMTs* and *TETs* mRNA levels were normalized to the same level as the control group by the end of the experiment.

In addition to gene expression analysis, we evaluated the activity of DNMTs and TETs in human PBMCs after exposure to THC. Our enzyme activity data aligned with our gene expression data. Over the course of five days, a THC concentration of 50 ng/ml did not significantly affect DNMTs or TETs activity (Supplementary Fig. 1B). However, we observed that time and THC exposure at a concentration of 200 ng/ml significantly affected DNMTs and TETs activity (Fig. 2B). Compared to the control group, DNMTs activity in the THC treatment group was significantly lower, while TETs activity was significantly greater. The most significant changes were observed 24 h after the third THC incubation ($p < 0.0001$; Šidak's post hoc test; $n = 16$). Both enzyme activities returned to the same level as the control group 24 h after the fifth and final incubation.

We continued the THCch study only at a THC concentration of 200 ng/ml. Our RT–qPCR findings demonstrate the notable effect of THC challenge incubation on cells previously exposed to THC (Fig. 3A). THCch incubation significantly decreased *DNMTs* mRNA levels compared to the control group (Kruskal–Wallis test: *DNMT1*, $p < 0.0001$; $n = 16$; one-way ANOVA main effect of the group: *DNMT3A*, $F_{(3,60)} = 35.94$, $p < 0.0001$; *DNMT3B*, $F_{(3,60)} = 56.36$, $p < 0.0001$; Tukey's post hoc test; $n = 16$). Conversely, after THCch incubation, *TETs* mRNA levels were significantly greater than those in the control group (Kruskal–Wallis test: *TET1*, *TET2*, $p < 0.0001$; $n = 16$; one-way ANOVA main effect of the group: *TET3*, $F_{(3,60)} = 116.4$, $p < 0.0001$; Tukey's post hoc test; $n = 16$).

The THCch incubation protocol produced a similar effect on DNMTs and TETs activity, as observed for gene expression (Fig. 3B). Compared to the control group, DNMTs activity in the treatment group was lower ($p < 0.0001$; Dunn's post hoc test; $n = 16$), and TETs activity was greater ($p < 0.01$; Dunn's post hoc test; $n = 16$).

R-THC and THCch incubations alter the gene expression of *CB1* and *CB2*

Once we confirmed that THC 200 ng/ml impacts the mRNA levels and enzyme activity of *DNMTs* and *TETs* in a concentration-dependent manner, we examined whether the mRNA levels of *CB1* and *CB2* (*CNR1* and *CNR2* genes) are similarly affected and whether these changes are connected to DNA methylation. First, we confirmed that *CB2* have a greater expression profile (CT values approximately 22–23) than *CB1* (CT values approximately 24–25) in PBMCs (data not shown).

In line with *DNMTs* and *TETs* results, repeated THC exposures at a concentration of 50 ng/ml did not produce any changes in *CB1* or *CB2* mRNA levels (Supplementary Fig. 1C), whereas time and a THC concentration of 200 ng/ml had significant effects (Fig. 2C). During the five-day period, both *CB1* and *CB2* mRNA levels increased gradually, with the peak level observed 24 h after the third incubation (*CB1*, $p < 0.0001$; Šidak's post hoc test; $n = 16$; *CB2*, $p < 0.0001$; Holm–Šidak's post hoc test; $n = 16$). By the end of the study period, 24 h after the fifth incubation, the mRNA levels of both receptors had normalized to the same level as the control group.

In the THCch experiment (Fig. 3C), compared to other groups, *CB1* and *CB2* mRNA levels were elevated in the THCch group (one-way ANOVA main effect of the group: *CB1*, $F_{(3,60)} = 28.34$, $p < 0.001$; *CB2*, $F_{(3,60)} = 31.84$, $p < 0.0001$; Tukey's post hoc test; $n = 16$).

R-THC and THCch caused genome-wide alterations in methylation and hydroxymethylation

After confirming that a high concentration of THC 200 ng/ml influences the gene expression and enzyme activity of *DNMTs* and *TETs* in human PBMCs in vitro, we investigated its effects at the genome-wide level. RRBS and RRoxBS sequencing were performed on three groups: "CTRL", "R-THC", and "THCch".

A total of 737 differentially methylated regions (DMRs) (381 hypermethylated, 356 hypomethylated) and 468 differentially hydroxymethylated regions (DhMRs) (310 hyperhydroxymethylated, 158 hypohydroxymethylated) were significantly associated with THC exposure between the R-THC group and the control group after correcting for multiple testing (Fig. 4A, B). After R-THC incubations, the ratio between hypermethylation and hypomethylation was balanced, but 66.24% of the differentially hydroxymethylated regions were hyperhydroxymethylated (Fig. 4E, F).

In the THCch group, a total of 693 DMRs (334 hypermethylated, 359 hypomethylated) and 442 DhMRs (174 hyperhydroxymethylated, 268 hypohydroxymethylated) were significantly associated with THC incubations compared to the control group after multiple testing was corrected (Fig. 4C, D). Again, the ratio of

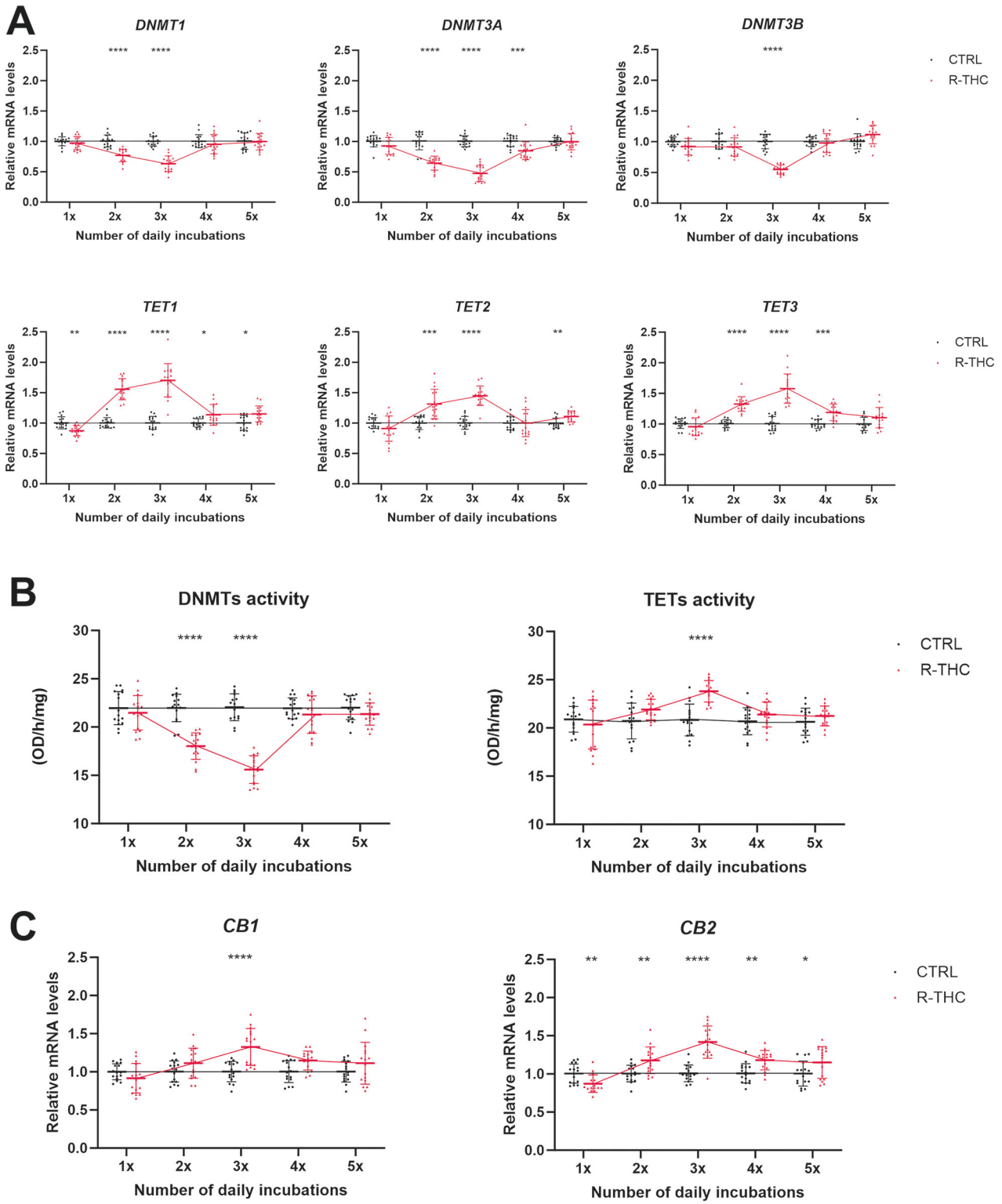


Fig. 2 Repeated high-concentration THC incubations cause alterations in epigenetic DNA modifiers and cannabinoid receptors. Repeated THC incubations. The cells were collected 24 h after every incubation. **A** mRNA levels of *DNMTs* and *TETs*; **B** enzyme activity of DNMTs and TETs; and **C** mRNA levels of *CB1* and *CB2* following repeated vehicle (CTRL) or THC (200 ng/ml) incubations (R-THC) in vitro in human PBMCs. Two-way repeated measures ANOVA followed by Šidak's post hoc test (*TET3, CB1*, DNMTs activity, and TETs activity); Mann–Whitney tests with Holm–Šidak's post hoc test (*DNMT1*, *DNMT3A*, *DNMT3B*, *TET1*, *TET2*, and *CB2*); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; $n = 16$. The error bars indicate SDs.

hypermethylated to hypomethylated regions was balanced, but exposure to THCch resulted in 60.63% of the differentially hydroxymethylated regions being hypohydroxymethylated (Fig. 4G, H).

Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) analyses

We performed GO and KEGG analyses to identify functionally relevant differentially methylated and hydroxymethylated genes

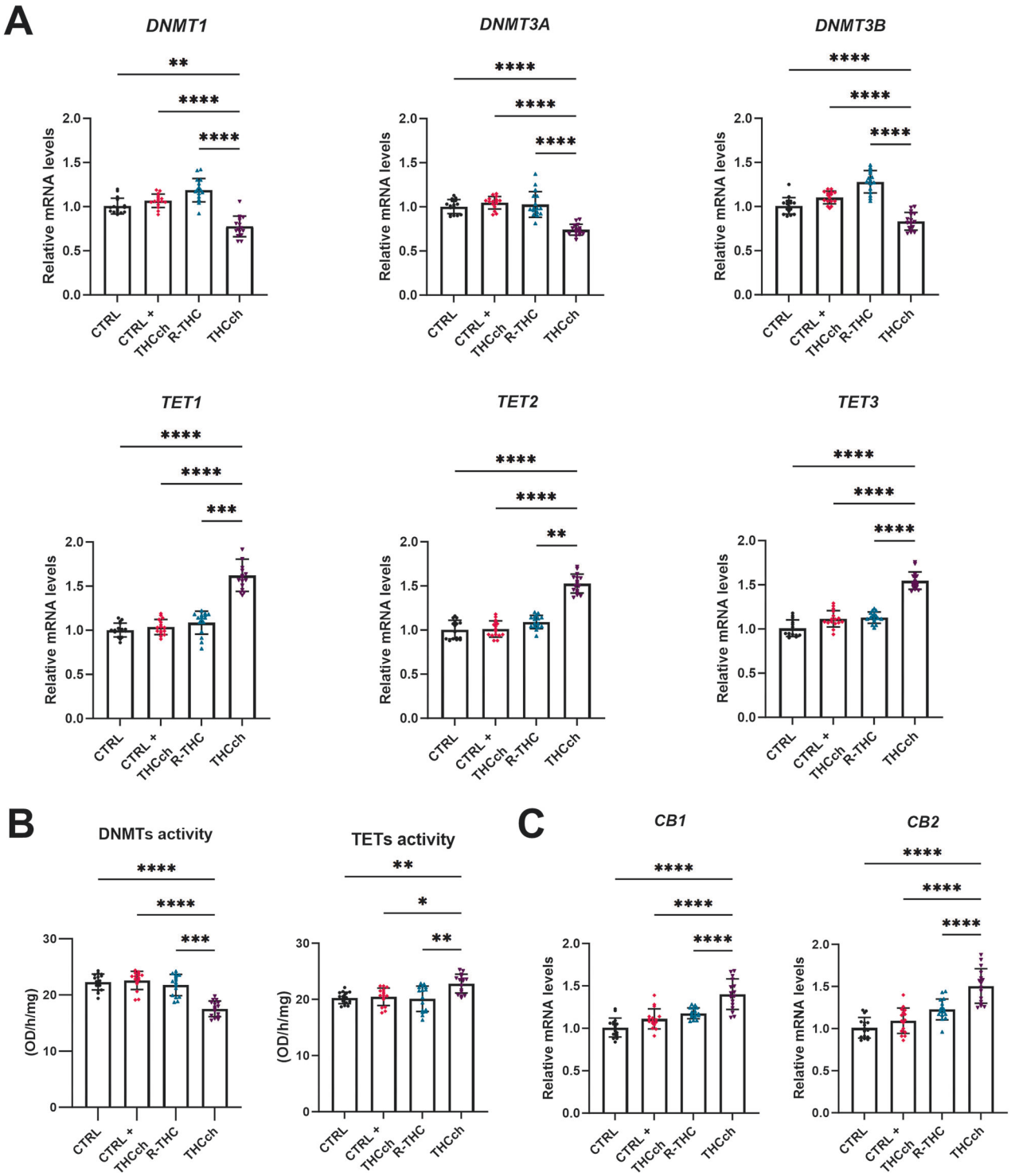
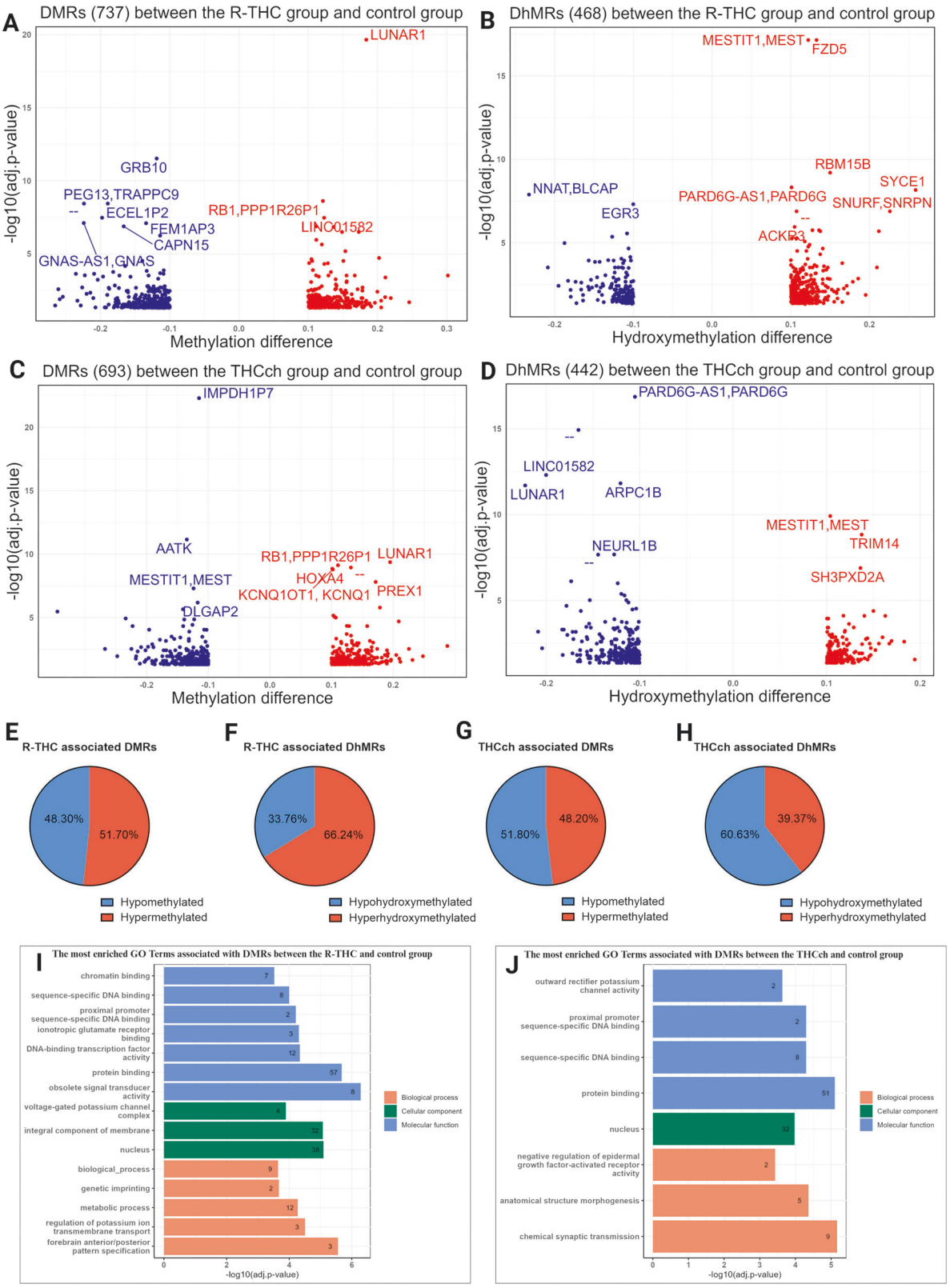


Fig. 3 High-concentration THC challenge incubation induces significant effects on epigenetic DNA modifiers and cannabinoid receptors in cells with prior exposure to THC. Repeated THC incubations + THC challenge (THCch). The cells were collected 24 h after the last challenge. **A** mRNA levels of *DNMTs* and *TETs*; **B** enzyme activity of DNMTs and TETs; **C** mRNA levels of *CB1* and *CB2* following repeated vehicle incubations + vehicle challenge (CTRL), repeated vehicle incubations + THC (200 ng/ml) challenge (CTRL+THCch), repeated THC (200 ng/ml) incubations + vehicle challenge (R-THC) and repeated THC (200 ng/ml) incubations + THC (200 ng/ml) challenge (THCch) in vitro in human PBMCs. One-way ANOVA followed by Tukey's post hoc test (*DNMT3A*, *DNMT3B*, *TET3*, *CB1*, and *CB2*); Kruskal–Wallis test followed by Dunn's post hoc test (*DNMT1*, *TET1*, *TET2*, DNMTs activity, and TETs activity); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; $n = 16$. The error bars indicate SDs.

associated with THC incubations. Our focus was on DMRs/DhMRs at the promoter regions because changes in methylation in gene bodies on the expression of genes are more complex than those in gene promoter regions.

In the R-THC group, 41 GO terms, and in the THCch group, 8 GO terms associated with differential methylation at the promoter regions after THC exposure remained significant (adj. $p < 0.05$) (Fig. 4I, J). In the R-THC group, the most enriched GO term was



obsolete signal transducer activity (adj. $p < 0.05$), and among the differentially methylated genes were those related to G-protein signaling, such as *AXIN1*, *GNAS*, and *GNAI2*. In the THCch group, the most enriched GO term was *chemical synaptic transmission* (adj. $p < 0.05$), and amongst the differentially methylated genes were *GABRB3*, *SLC6A2*, *KCNK2*, *KCNB1*, *CACNA1E*, and *CACNG8*. A detailed list of GO terms and differentially methylated genes associated with THC incubations can be found in Supplementary Table 8.

Table 1 presents the KEGG pathways enriched with genes associated with differential methylation at the promoter regions

Fig. 4 Differential methylation and hydroxymethylation associated with high-concentration THC exposure. A Differentially methylated regions (DMRs) and **B** differentially hydroxymethylated regions (DhMRs) between the repeated THC (200 ng/ml) incubations group (R-THC) and the control group. **C** DMRs and **D** DhMRs between the repeated THC (200 ng/ml) incubations + THC challenge (200 ng/ml) group (THCch) and the control group. After adjusting for multiple comparisons, annotations are displayed for the ten most statistically significant DMRs/DhMRs. The y-axis shows the –log10(adjusted p-value), and the x-axis represents the difference in methylation/hydroxymethylation (red – hypo(hydroxy)methylated, blue – hyper(hydroxy)methylated). **E** Hypermethylation and hypomethylation among the R-THC-associated DMRs. **F** Hyperhydroxymethylation and hypohydroxymethylation among the R-THC-associated DhMRs. **G** Hypermethylation and hypomethylation among the THCch-associated DMRs. **H** Hyperhydroxymethylation and hypohydroxymethylation among the THCch-associated DhMRs. **I** Gene Ontology (GO) analysis of genes associated with differential methylation at promoter regions after THC exposure between the repeated THC (200 ng/ml) incubations group (R-THC) and the control group. **J** GO analysis of genes associated with differential methylation at the promoter regions after THC exposure between the repeated THC (200 ng/ml) incubations + THC challenge (200 ng/ml) group (THCch) and the control group. Adjusted *p*-value < 0.05, if more than 15 significant entries than 15 most significant GO terms are displayed. The number on the horizontal bar indicates the number of enriched genes in the GO term.

Table 1. KEGG pathways enriched with genes associated with differential methylation at promoter regions after THC exposure.

Term:	Fold change:	Adjusted p-value:	Pathway:	Gene list:
R-THC group vs control group:				
hsa05200	6.336011	0.006850595	Pathways in cancer	NTRK1;AXIN1;CREBBP;GNAI2;GNAS;RALGDS;CBLC;FOXO1;
hsa04950	37.540826	0.006850595	Maturity onset diabetes of the young	HNF1B;NKX2-2;PAX6;
hsa05032	13.904048	0.011635664	Morphine addiction	GABRB3;GNAS;PDE1C;GNAI2;
hsa04750	12.640028	0.012360714	Inflammatory mediator regulation of TRP channels	IL1R1;NTRK1;GNAS;ALOX12;
hsa04726	11.074013	0.015991563	Serotonergic synapse	GABRB3;GNAS;ALOX12;GNAI2;
hsa04730	15.642027	0.025426552	Long-term depression	GNAS;RYR1;GNAI2;
hsa04924	14.438765	0.027174152	Renin secretion	GNAS;PDE1C;GNAI2;
hsa05202	7.030124	0.043917513	Transcriptional misregulation in cancer	NTRK1;FLT1;HIST1H3E;FOXO1;
hsa05034	6.990856	0.043917513	Alcoholism	GNAI2;HIST1H3E;GNAS;HIST1H2AJ;
hsa04713	9.776286	0.043917513	Circadian entrainment	GNAS;RYR1;GNAI2;
hsa05169	6.28825	0.043917513	Epstein-Barr virus infection	POLR2L;SPN;CREBBP;HSPA1L;
hsa04915	9.385206	0.043917513	Estrogen signaling pathway	HSPA1L;GNAS;GNAI2;
hsa04922	9.385206	0.043917513	Glucagon signaling pathway	FOXO1;GNAS;CREBBP;
hsa04916	9.292299	0.043917513	Melanogenesis	GNAS;CREBBP;GNAI2;
hsa00512	20.183264	0.043917513	Mucin type O-Glycan biosynthesis	WBSCR17;GALNT6;
hsa04015	5.987379	0.043917513	Rap1 signaling pathway	RALGDS;FLT1;GNAI2;GNAS;
THC challenge (THCch) group vs control group:				
hsa03018	18.272433	0.008912327	RNA degradation	PFKP;PABPC1L2A;PABPC3;PABPC5;
hsa03015	15.430075	0.008912327	mRNA surveillance pathway	PABPC1L2A;PABPC3;SSU72;PABPC5;
hsa04010	6.807389	0.036304807	MAPK signaling pathway	FGF3;FGF11;CACNA1E;CACNG8;MAP4K4;

after THC incubations. In the R-THC group, 16 KEGG pathways remained statistically significant, and the most significantly enriched pathway was *pathways in cancer* (adj. $p < 0.05$). Among the differentially methylated genes were *GNAS*, *GNAI2*, and genes necessary for the regulation of the transcription of *CREBBP* and *FOXO1*. In the THCch group, three pathways remained significantly different: *RNA degradation*, *the mRNA surveillance pathway*, and the *MAPK signaling pathway* (adj. $p < 0.05$).

GO analysis of genes with differential hydroxymethylation after THC incubations revealed six significantly enriched GO terms in the R-THC group, with *protein secretion* being the most significantly enriched GO term (adj. $p < 0.05$). In the THCch group, we identified one statistically significant GO term, *response to fibroblast growth factor* (adj. $p < 0.05$). The detailed lists can be found in Supplementary Table 9. KEGG pathway analysis of the DhMR-annotated genes did not reveal significantly enriched pathways (adj. $p < 0.05$).

The GO terms and KEGG pathways enriched with genes associated with DMRs/DhMRs in the gene bodies after R-THC and THCch incubation can be found in Supplementary Tables 10–12.

Based on the statistically significant DMR/DhMR-related enrichment at the promoter region, we selected four genes, *GNAS, FOXO1*, *IL1R1*, and *CD93*, for qPCR gene expression validation. Our results showed that changes in DNA modifications correlated with changes in the mRNA levels of these genes (Supplementary Fig. 2).

R-THC and THCch incubations alter the expression of genes related to cytokine production

We studied how 50 and 200 ng/ml THC modulates the immune system, particularly whether it leads to aberrant cytokine production. We examined the expression of genes that encode the pro-inflammatory cytokines *IL-1β*, *IL-6*, and *TNF-α* and the anti-inflammatory cytokine *IL-10* (Fig. 5A, B). Surprisingly, the changes in pro-inflammatory or anti-inflammatory cytokine-encoding genes were not dependent on the THC concentration. Our findings showed that daily incubations with THC at both concentrations (50 and 200 ng/ml) increased the mRNA levels of

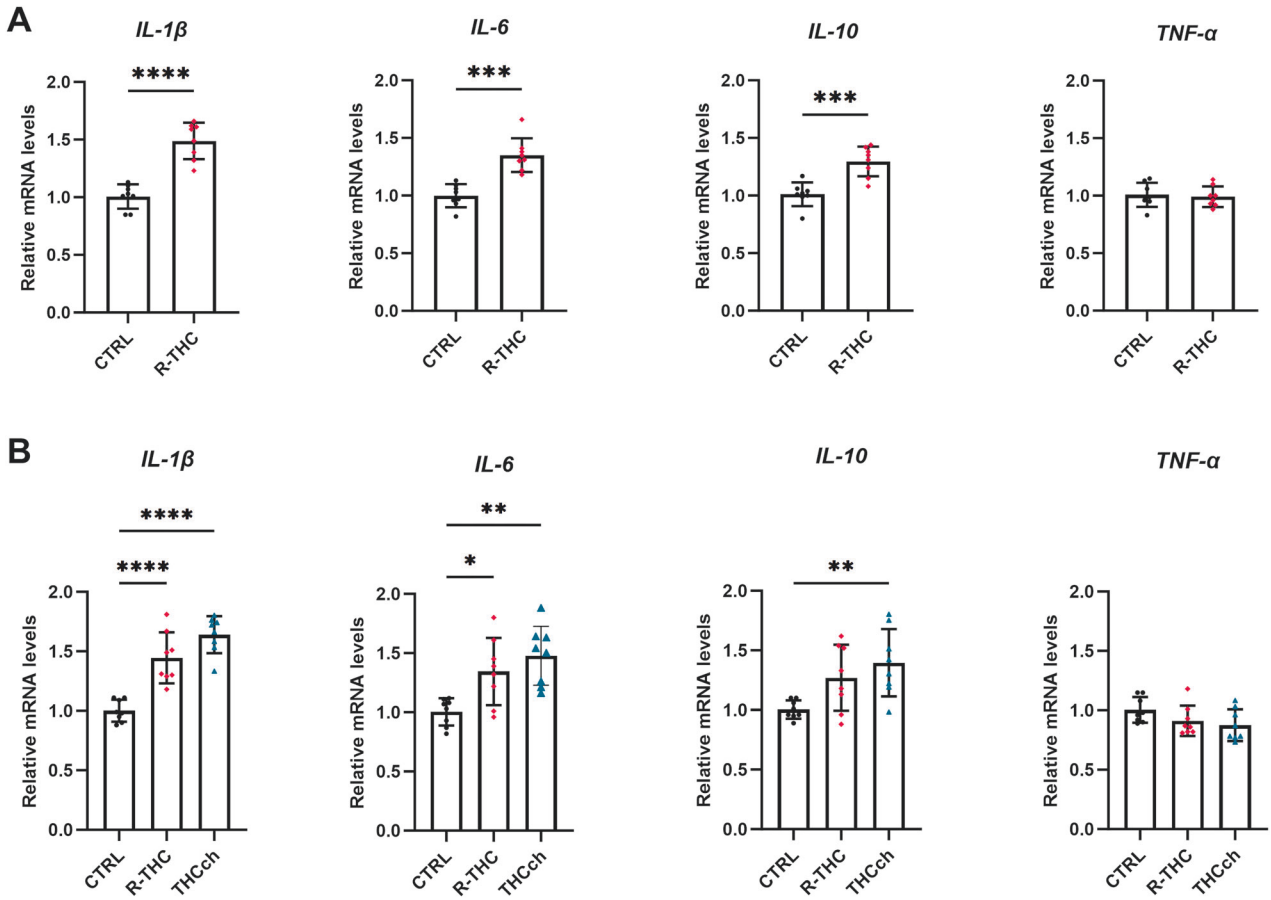


Fig. 5 Low and high concentration of THC alter the expression of genes related to cytokine production. A mRNA levels of *IL-1β*, *IL-6*, *IL-10*, and *TNF-α* in PBMCs following repeated vehicle (CTRL) or THC (50 ng/ml) incubations (R-THC) in vitro. Student's t-test. **B** mRNA levels of *IL-1β*, *IL-6*, *IL-10*, and *TNF-α* in PBMCs following repeated vehicle incubations + vehicle challenge (CTRL), repeated THC (200 ng/ml) incubations + vehicle challenge (R-THC) or repeated THC (200 ng/ml) incubations + THC (200 ng/ml) challenge (THCch) in vitro. One-way ANOVA followed by Tukey's post hoc test (for *IL-1β, IL-6*, and *IL-10)*; Kruskal–Wallis test followed by Dunn's post hoc test (for *TNF-α*); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; $n = 8$. The error bars indicate SDs.

the pro-inflammatory cytokines *IL-1β* and *IL-6* and the anti-inflammatory cytokine *IL-10* compared to the control group. After five consecutive days of incubations with 50 ng/ml THC, the *IL-1β*, *IL-6*, and *IL-10* mRNAs were upregulated 24 h after the last incubation (Student's t-test: *IL-1β*, $t = 7.160$, $p < 0.0001$; *IL-6*, $t = 5.627$, $p < 0.001$; *IL-10*, $t = 4.879$, $p < 0.001$; CTRL vs THC 50 ng/ml; $n = 8$). A concentration of 200 ng/ml similarly upregulated the *IL-1β*, *IL-6*, and *IL-10* mRNA levels 24 h after the fifth daily incubation. An additional THCch treatment further increased the mRNA levels (one-way ANOVA main effect of the group: *IL-1β*, $F_{(2,21)} = 32.62$, $p < 0.0001$; *IL-6*, $F_{(2,21)} = 9.161$, $p < 0.01$; and *IL-10*, $F_{(2,21)} = 5.934$, $p < 0.01$; Tukey's post hoc test; $n = 8$). However, THC incubations on five consecutive days and an additional incubation after a period of abstinence did not affect the mRNA levels of the *TNF-α* gene.

DISCUSSION

A critical question related to CUD is why some occasional cannabis users become addicted and what the underlying mechanism of such a change is. We hypothesize that this may be due to a combination of genetic predisposition [34], a critical period for brain development [35, 36], and high concentrations of THC in the body [37]. We speculate that a high concentration of THC-induced aberrant DNA methylation may underlie long-term changes in gene expression, which in turn may be one of the factors contributing to the development of CUD. This study emphasizes the dose-dependent effect of THC on the enzymatic activity of DNMTs and TETs, which is supported by prior studies that link DNA methylation to the interplay between cannabis use and gene expression changes [19, 38]. Additionally, some changes in DNA methylation induced by environmental factors can be long-term and, consequently, have a lasting effect on gene expression, whereas others are dynamic and transient [39, 40]. Our results do not exclude the possibility that posttranslational histone modifications and microRNAs may also be involved in THC-induced alterations in gene expression.

First, we investigated how repeated incubations with THC at concentrations of 50 and 200 ng/ml regulate *DNMTs* and *TETs* mRNA levels and enzymatic activity in PBMCs. Acute incubation with THC at 50 or 200 ng/ml did not affect the mRNA levels or enzyme activity of DNMTs and TETs. These findings support our earlier finding that acute treatment with cocaine and amphetamine did not affect *DNMTs* and *TETs* expression in human PBMCs [25], indicating that acute exposure to environmental factors does not affect epigenetic DNA modifiers.

To assess the long-term effects of THC incubations on *DNMTs* and *TETs*, we collected PBMCs 24 h after incubations. Repeated 50 ng/ml THC did not affect *DNMTs* and *TETs* mRNA levels or enzyme activity, while dynamic changes occurred after repeated incubations with 200 ng/ml THC. We found significant alterations in *DNMTs* and *TETs* mRNA levels and enzyme activity after three days of repeated incubations with THC at 200 ng/ml.

The findings of this study demonstrated that repeated incubations with 200 ng/ml THC can induce tolerance, as we observed a decrease in *CB1, CB2* and *TETs* mRNA levels on the 4 and 5th THC incubation days compared with the maximum THC effect on the 3rd day in PBMCs. The development of tolerance may be attributed to a decrease in the activity of intracellular signaling originating from cannabinoid receptors and/or a decrease in the number of cannabinoid receptors on the plasma membrane [41]. Like in previous studies, our THC challenge experiments showed that tolerance can be reversed through a period of drug abstinence [42]. However, the results also demonstrated the normalization of *DNMTs* mRNA levels on the 4th and 5th days after THC incubation, suggesting that this normalization could be the result of mutual regulation between DNMTs and TETs.

Our in vitro THC-induced changes in the gene expression of epigenetic DNA modifiers and CB1 and CB2 receptors did not replicate many of the findings in chronic cannabis users. This discrepancy may be due to the additional components of cannabis cigarettes compared to those of pure THC, the investigational time point chosen, or various environmental factors to which cannabis users may be exposed. Specifically, Smith and colleagues assessed the acute effects of cannabis cigarette smoking (55 min, 1 h 20 min, and 4 h later) on *DNMTs*, *TETs*, *CB1* and *CB2* mRNA levels in chronic marijuana smokers [22]. Although their experimental design resembles the challenge experiment in our study, we studied THC-induced changes in epigenetic DNA editors in PBMCs 24 h later to assess persistent changes. Therefore, our findings are not in conflict with the aforementioned study, as both investigations evaluated the effects of THC at distinct time intervals. Other studies also suggest that the effect of marijuana smoking on DNA methylation can be extensive, with several factors potentially influencing the outcome. For instance, Fang and others speculated that cannabis use and cigarette smoking could independently influence DNA methylation levels, possibly due to shared common combustible chemicals [19]. These findings indicate that the effects of marijuana smoking and THC incubations on epigenetic DNA modifiers may vary and depend on co-factors.

Our findings corroborate prior research showing that PBMCs express *CB2* at higher levels than *CB1* [22, 43]. Intriguingly, after repeated doses of 200 ng/ml THC, the mRNA levels of both *CB1* and *CB2* shifted in a manner consistent with TETs enzyme activity. Several studies have shown potential crosstalk between endocannabinoid system activity and DNA methylation modifier enzymes. For instance, THC-dependent patients exhibit reduced methylation of the *CB1* gene promoter and increased *CB1* mRNA levels in peripheral blood cells [23]. Similarly, increased *CB1* gene mRNA levels are associated with reduced DNA methylation at the *CB1* gene promoter in schizophrenic patients [44]. Compared to DNA methylation mechanisms, THC-induced TETs enzyme activity has been less studied; therefore, its effects remain largely unclear. We speculate that the activity of the PI3K-Akt and ERK1/2 signaling pathways following CB1 and CB2 stimulation may modulate TETs activity in PBMCs.

In subsequent investigations, we explored how THC at 200 ng/ml affects genome-wide DNA methylation and hydroxymethylation in human PBMCs. THC incubations led to a balanced ratio between differential hypermethylation and hypomethylation. However, after repeated THC incubations, the differentially hydroxymethylated genes mostly contained hyperhydroxymethylated regions. Interestingly, even though we observed increased TETs activity after THCch treatment, at the genome-wide level, more genes contained hypohydroxymethylated regions. One possible explanation may be that as 5-hmC is the first step in the demethylation process, the increased activity of TETs causes greater conversion of 5-hmC to subsequent 5-formylcytosine and 5-carboxylcytosine and, ultimately, their replacement with unmodified cytosine.

GO and KEGG pathway analyses revealed that the observed alterations in DNA methylation and hydroxymethylation only partially replicated the findings of previous studies. We found differential methylation of the G protein-related *GNAS* gene in both the R-THC and THCch groups compared to the control group. The *GNAS* gene was enriched in multiple GO terms and KEGG pathways and encodes the alpha-subunit of guanine nucleotide-binding protein, which mediates the actions of many hormones and neurotransmitters through the signal transduction process [45].

In the THCch group, the most enriched GO term was *chemical synaptic transmission*. Among the differentially methylated genes was the calcium channel subunit encoding gene (*CACNA1E*), which was hypermethylated in promoter regions. Previous studies have linked aberrant DNA methylation to multiple psychiatric disorders [46, 47], and various constituents of cannabis are being researched for the treatment of epilepsy by targeting calcium signaling and *MAPK signaling pathways*, among others [48]. The *MAPK signaling pathway* remained significantly different in our study, which aligns with the findings of other epigenome-wide studies on cannabis use [17, 49]. Abnormal activity of the *MAPK signaling pathway* has been implicated in the pathogenesis of schizophrenia [50], and cannabis use, especially at higher THC concentrations, is associated with immunomodulating properties and an increased risk of psychosis [51].

We validated the expression of four genes (*GNAS, FOXO1, IL1R1*, and *CD93*) that exhibited significant DMRs/DhMRs at the promoter region and found that these changes correlated with alterations in the mRNA levels of these genes. Although we also observed significant changes in the mRNA levels of *DNMTs*, *TETs*, *CB1*, and *CB2* genes in the THCch 200 ng/ml treatment group, these changes did not manifest in the DMR/DhMR findings. This finding suggested that, in this model *DNMTs*, *TETs*, *CB1*, and *CB2* expression changes are not solely regulated by DNA methylation. We speculate that THC incubations cause a dose-dependent increase in TETs activity and elevated 5-hmC levels in PBMCs. 5-hmc is not only an intermediate of DNA demethylation but also has distinct epigenetic regulatory functions. For example, 5-hmC is associated with activating the histone marks H3K4me1, H3K4me3, and H3K27ac [52–54], causing chromatin to open, thereby promoting gene transcription. The changes in *DNMTs*, *TETs*, and *CB1/2* receptor expression observed after THCch may result from a combination of DNA hydroxymethylation and histone modifications. These THCch effects can persist and act as epigenetic priming [55], where changes in DNA methylation and chromatin modifications in the promoter region facilitate gene expression. Previous studies suggest that epigenetic priming may be a key mechanism underlying long-lasting alterations in gene regulation [56], which can remain latent until triggered by re-exposure to drug-associated stimuli or the drug itself [55].

THC affects signal transduction, alters synaptic plasticity, and is implicated in immunomodulatory responses [6, 57–60]. This led us to examine the relationship between repeated THC (50 and 200 ng/ml) incubations and the immune response. Our study revealed that repeated incubations with both 50 and 200 ng/ml THC led to increased mRNA levels of *IL-6* and *IL-1β*, consistent with prior findings of elevated pro-inflammatory cytokines in marijuana users [61–63]. No change in *TNF-α* mRNA levels was observed following repeated THC incubations. Interestingly, exposure to THC at both concentrations also increased the *IL-10* mRNA level, suggesting the activation of anti-inflammatory pathways upon CB1 and CB2 stimulation. After the abstinence period, THCch incubation further elevated the mRNA levels of both pro-inflammatory and anti-inflammatory cytokines compared to the control and repeated THC incubations groups. Although previous research has indicated that DNA methylation changes can regulate *IL-6*, *IL-10*, and *IL-1β* gene expression [64–66], our DMR/DhMR analysis did not detect any methylation or hydroxymethylation changes in the promoter regions of these genes in PBMCs. Therefore, the

upregulation of the expression of these cytokine-encoding genes following repeated THC incubations was not due to aberrant DNA methylation, suggesting that other regulatory mechanisms influence the changes in the expression of these genes.

CONCLUSIONS

Our findings demonstrated that repeated incubations with THC at 200 ng/ml resulted in alterations in the mRNA levels of *DNMTs* and *TETs*, as well as in the enzyme activities in PBMCs. At the genome-wide level, repeated incubations with THC led to increased hydroxymethylation. Following the THC challenge, the hyperhydroxymethylated state shifted to hypohydroxymethylation. These findings suggest that repeated incubations with high concentrations of THC induce genome-wide demethylation, potentially affecting the expression of genes critical for the development of CUD. However, the aberrant DNA methylation observed in our in vitro studies with THC partially differs from previous DNA methylation results obtained in studies of cannabis users, which may limit the biological significance of these changes.

DATA AVAILABILITY

The raw RRBS data sets were deposited in the SRA BioProject under the accession number PRJNA1190049. Extended results for DMRs/DhMRs are available in the Figshare repository https://doi.org/10.6084/m9.figshare.27653313. Additional data is available from the corresponding author upon reasonable request.

REFERENCES

1. World Drug Report 2024. United Nations: Office on Drugs and Crime. 2024. www.unodc.org/unodc/en/data-and-analysis/world-drug-report-2024.html (accessed 9 Jun 2025).
2. Connor JP, Stjepanović D, Le Foll B, Hoch E, Budney AJ, Hall WD. Cannabis use and cannabis use disorder. Nat Rev Dis Primers. 2021;7:1–24.
3. Lopez-Quintero C, Pérez de los Cobos J, Hasin DS, Okuda M, Wang S, Grant BF, et al. Probability and predictors of transition from first use to dependence on nicotine, alcohol, cannabis, and cocaine: results of the national epidemiologic survey on alcohol and related conditions (NESARC). Drug Alcohol Depend. 2011;115:120–30.
4. Hasin DS. US epidemiology of cannabis use and associated problems. Neuropsychopharmacology. 2018;43:195–212.
5. Atakan Z. Cannabis, a complex plant: different compounds and different effects on individuals. Ther Adv Psychopharmacol. 2012;2:241–54.
6. Klein TW, Newton C, Larsen K, Lu L, Perkins I, Nong L, et al. The cannabinoid system and immune modulation. J Leukoc Biol. 2003;74:486–96.
7. Mechoulam R, Parker LA. The endocannabinoid system and the brain. Annu Rev Psychol. 2013;64:21–47.
8. Cadet JL, Jayanthi S. Epigenetics of addiction. Neurochem Int. 2021;147:105069.
9. Cecil CAM, Walton E, Viding E. DNA methylation, substance use and addiction: a systematic review of recent animal and human research from a developmental perspective. Curr Addict Rep. 2015;2:331–46.
10. Nestler EJ. Epigenetic mechanisms of drug addiction. Neuropharmacology. 2014;76:259–68.
11. Goll MG, Bestor TH. Eukaryotic cytosine methyltransferases. Annu Rev Biochem. 2005;74:481–514.
12. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. 2003;33:245–54.
13. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. Science. 2011;333:1300.
14. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009;324:930–5.
15. Szutorisz H, Hurd YL. Epigenetic effects of cannabis exposure. Biol Psychiatry. 2016;79:586–94.
16. Markunas CA, Hancock DB, Xu Z, Quach BC, Fang F, Sandler DP, et al. Epigenome-wide analysis uncovers a blood-based DNA methylation biomarker of lifetime cannabis use. Am J Med Genet B Neuropsychiatr Genet. 2021;186:173–82.
17. Nannini DR, Zheng Y, Joyce BT, Kim K, Gao T, Wang J, et al. Genome-wide DNA methylation association study of recent and cumulative marijuana use in middle aged adults. Mol Psychiatry. 2023;28:2572–82.
18. Osborne AJ, Pearson JF, Noble AJ, Gemmell NJ, Horwood LJ, Boden JM, et al. Genome-wide DNA methylation analysis of heavy cannabis exposure in a New Zealand longitudinal cohort. Transl Psychiatry. 2020;10:114.
19. Fang F, Quach B, Lawrence KG, van Dongen J, Marks JA, Lundgren S, et al. Trans-ancestry epigenome-wide association meta-analysis of DNA methylation with lifetime cannabis use. Mol Psychiatry 2024;29:124–133.
20. Wiedmann M, Kuitunen-Paul S, Basedow LA, Wolff M, DiDonato N, Franzen J, et al. DNA methylation changes associated with cannabis use and verbal learning performance in adolescents: an exploratory whole genome methylation study. Transl Psychiatry. 2022;12:1–10.
21. Fuchs Weizman N, Wyse BA, Montbriand J, Jahangiri S, Librach CL. Cannabis significantly alters DNA methylation of the human ovarian follicle in a concentration-dependent manner. Mol Hum Reprod. 2022;28:gaac022.
22. Smith RC, Sershen H, Janowsky DS, Lajtha A, Grieco M, Gangoiti JA, et al. Changes in expression of DNA-methyltransferase and cannabinoid receptor mRNAs in blood lymphocytes after acute cannabis smoking. Front Psychiatry. 2022;13:887700.
23. Rotter A, Bayerlein K, Hansbauer M, Weiland J, Sperling W, Kornhuber J, et al. CB1 and CB2 receptor expression and promoter methylation in patients with cannabis dependence. Eur Addict Res. 2012;19:13–20.
24. Anier K, Urb M, Kipper K, Herodes K, Timmusk T, Zharkovsky A, et al. Cocaine-induced epigenetic DNA modification in mouse addiction-specific and non-specific tissues. Neuropharmacology. 2018;139:13–25.
25. Anier K, Somelar K, Jaako K, Alttoa M, Sikk K, Kokassaar R, et al. Psychostimulant-induced aberrant DNA methylation in an in vitro model of human peripheral blood mononuclear cells. Clinical Epigenetics 2022; 14. https://doi.org/10.1186/s13148-022-01303-w.
26. Buttarelli FR, Fanciulli A, Pellicano C, Pontieri FE. The dopaminergic system in peripheral blood lymphocytes: from physiology to pharmacology and potential applications to neuropsychiatric disorders. Curr Neuropharmacol. 2011;9:278–88.
27. Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, et al. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. Eur J Biochem. 1995;232:54–61.
28. Namba MD, Leyrer-Jackson JM, Nagy EK, Olive MF, Neisewander JL. Neuroimmune mechanisms as novel treatment targets for substance use disorders and associated comorbidities. Front Neurosci. 2021;15:650785.
29. Fabritius M, Chtioui H, Battistella G, Annoni J-M, Dao K, Favrat B, et al. Comparison of cannabinoid concentrations in oral fluid and whole blood between occasional and regular cannabis smokers prior to and after smoking a cannabis joint. Anal Bioanal Chem. 2013;405:9791–803.
30. Hunault CC, Mensinga TT, de Vries I, Kelholt-Dijkman HH, Hoek J, Kruidenier M, et al. Delta-9-tetrahydrocannabinol (THC) serum concentrations and pharmacological effects in males after smoking a combination of tobacco and cannabis containing up to 69 mg THC. Psychopharmacology. 2008;201:171–81.
31. Marsot A, Audebert C, Attolini L, Lacarelle B, Micallef J, Blin O. Comparison of cannabinoid concentrations in plasma, oral fluid and urine in occasional cannabis smokers after smoking cannabis cigarette. J Pharm Pharm Sci. 2016;19:411–22.
32. Ramaekers JG, Moeller MR, van Ruitenbeek P, Theunissen EL, Schneider E, Kauert G. Cognition and motor control as a function of Δ9-THC concentration in serum and oral fluid: limits of impairment. Drug Alcohol Depend. 2006;85:114–22.
33. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3:1101–8.
34. Goldman D, Oroszi G, Ducci F. The genetics of addictions: uncovering the genes. Nat Rev Genet. 2005;6:521–32.
35. Wittchen H-U, Fröhlich C, Behrendt S, Günther A, Rehm J, Zimmermann P, et al. Cannabis use and cannabis use disorders and their relationship to mental disorders: A 10-year prospective-longitudinal community study in adolescents. Drug Alcohol Depend. 2007;88:S60–S70.
36. Hurd YL, Manzoni OJ, Pletnikov MV, Lee FS, Bhattacharyya S, Melis M. Cannabis and the developing brain: insights into its long-lasting effects. J Neurosci. 2019;39:8250–8.
37. Ferland J-MN, Ellis RJ, Rompala G, Landry JA, Callens JE, Ly A, et al. Dose mediates the protracted effects of adolescent THC exposure on reward and stress reactivity in males relevant to perturbation of the basolateral amygdala transcriptome. Mol Psychiatry. 2023;28:2583–93.
38. Nannini DR, Zheng Y, Joyce BT, Gao T, Liu L, Jacobs DR, et al. Marijuana use and DNA methylation-based biological age in young adults. Clin Epigenetics. 2022;14:134.
39. Kaur G, Begum R, Thota S, Batra S. A systematic review of smoking-related epigenetic alterations. Arch Toxicol. 2019;93:2715–40.
40. Feil R, Fraga MF. Epigenetics and the environment: emerging patterns and implications. Nat Rev Genet. 2012;13:97–109.
41. Piscura MK, Henderson-Redmond AN, Barnes RC, Mitra S, Guindon J, Morgan DJ. Mechanisms of cannabinoid tolerance. Biochem Pharmacol. 2023;214:115665.
42. MacCallum CA, Russo EB. Practical considerations in medical cannabis administration and dosing. Eur J Intern Med. 2018;49:12–19.

43. Muhl D, Kathmann M, Hoyer C, Kranaster L, Hellmich M, Gerth CW, et al. Increased CB2 mRNA and anandamide in human blood after cessation of cannabis abuse. Naunyn Schmiedebergs Arch Pharmacol. 2014;387:691–5.
44. D'Addario C, Micale V, Di Bartolomeo M, Stark T, Pucci M, Sulcova A, et al. A preliminary study of endocannabinoid system regulation in psychosis: distinct alterations of *CNR1* promoter DNA methylation in patients with schizophrenia. Schizophr Res. 2017;188:132–40.
45. Weinstein LS, Xie T, Zhang Q-H, Chen M. Studies of the regulation and function of the Gsα gene Gnas using gene targeting technology. Pharmacol Ther. 2007;115:271–91.
46. Andrade A, Brennecke A, Mallat S, Brown J, Gomez-Rivadeneira J, Czepiel N, et al. Genetic Associations between voltage-gated calcium channels and psychiatric disorders. Int J Mol Sci. 2019;20:3537.
47. Li W, Fan CC, Mäki-Marttunen T, Thompson WK, Schork AJ, Bettella F, et al. A molecule-based genetic association approach implicates a range of voltage-gated calcium channels associated with schizophrenia. Am J Med Genet B Neuropsychiatr Genet. 2018;177:454–67.
48. Li Y, Ding Y, Xiao W, Zhu J-B. Investigation on the active ingredient and mechanism of *Cannabis sativa* L. for treating epilepsy based on network pharmacology. Biotechnol Biotechnol Equip. 2021;35:994–1009.
49. Murphy SK, Itchon-Ramos N, Visco Z, Huang Z, Grenier C, Schrott R, et al. Cannabinoid exposure and altered DNA methylation in rat and human sperm. Epigenetics. 2018;13:1208–21.
50. Funk AJ, McCullumsmith RE, Haroutunian V, Meador-Woodruff JH. Abnormal activity of the MAPK- and cAMP-associated signaling pathways in frontal cortical areas in postmortem brain in schizophrenia. Neuropsychopharmacology. 2012;37:896–905.
51. Gibson CL, Bassir Nia A, Spriggs SA, DeFrancisco D, Swift A, Perkel C, et al. Cannabinoid use in psychotic patients impacts inflammatory levels and their association with psychosis severity. Psychiatry Res. 2020;293:113380.
52. Tsagaratou A, Äijö T, Lio C-WJ, Yue X, Huang Y, Jacobsen SE, et al. Dissecting the dynamic changes of 5-hydroxymethylcytosine in T-cell development and differentiation. Proc Natl Acad Sci USA. 2014;111:E3306–E3315.
53. Pastor WA, Pape UJ, Huang Y, Henderson HR, Lister R, Ko M, et al. Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature. 2011;473:394–7.
54. Stroud H, Feng S, Morey Kinney S, Pradhan S, Jacobsen SE. 5-Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem cells. Genome Biol. 2011;12:R54.
55. Mews P, Walker DM, Nestler EJ. Epigenetic priming in drug addiction. Cold Spring Harb Symp Quant Biol. 2019;83:131.
56. Kundakovic M, Chen Y, Guidotti A, Grayson DR. The reelin and GAD67 promoters are activated by epigenetic drugs that facilitate the disruption of local repressor complexes. Mol Pharmacol. 2008;75:342.
57. Morcuende A, García-Gutiérrez MS, Tambaro S, Nieto E, Manzanares J, Femenia T Immunomodulatory role of cb2 receptors in emotional and cognitive disorders. Front Psychiatry 2022; 13. https://doi.org/10.3389/fpsyt.2022.866052.
58. Fan N, Yang H, Zhang J, Chen C. Reduced expression of glutamate receptors and phosphorylation of CREB are responsible for in vivo Δ9-THC exposure-impaired hippocampal synaptic plasticity. J Neurochem. 2010;112:691–702.
59. Henshaw FR, Dewsbury LS, Lim CK, Steiner GZ. The effects of cannabinoids on pro- and anti-inflammatory cytokines: a systematic review of in vivo studies. Cannabis Cannabinoid Res. 2021;6:177–95.
60. Mato S, Chevaleyre V, Robbe D, Pazos A, Castillo PE, Manzoni OJ. A single exposure to Δ9THC blocks endocannabinoid-mediated synaptic plasticity. Nat Neurosci. 2004;7:585–6.
61. Saroz Y, Kho DT, Glass M, Graham ES, Grimsey NL. Cannabinoid receptor 2 (CB2) signals via g-alpha-s and induces IL-6 and IL-10 cytokine secretion in human primary leukocytes. ACS Pharmacol Transl Sci. 2019;2:414–28.
62. Bayazit H, Selek S, Karababa IF, Cicek E, Aksoy N. Evaluation of oxidant/antioxidant status and cytokine levels in patients with cannabis use disorder. Clin Psychopharmacol Neurosci. 2017;15:237–42.
63. Berdyshev EV, Boichot E, Germain N, Allain N, Anger J-P, Lagente V. Influence of fatty acid ethanolamides and Δ9-tetrahydrocannabinol on cytokine and arachidonate release by mononuclear cells. Eur J Pharmacol. 1997;330:231–40.
64. Tekpli X, Landvik NE, Anmarkud KH, Skaug V, Haugen A, Zienolddiny S. DNA methylation at promoter regions of interleukin 1B, interleukin 6, and interleukin 8 in non-small cell lung cancer. Cancer Immunol Immunother. 2012;62:337–45.
65. Tang B, Zhao R, Sun Y, Zhu Y, Zhong J, Zhao G, et al. Interleukin-6 expression was regulated by epigenetic mechanisms in response to influenza virus infection or dsRNA treatment. Mol Immunol. 2011;48:1001–8.
66. Alipour S, Nouri M, Khabbazi A, Samadi N, Babaloo Z, Abolhasani S, et al. Hypermethylation of IL-10 gene is responsible for its low mRNA expression in Behçet's disease. J Cell Biochem. 2018;119:6614–22.

AUTHOR CONTRIBUTIONS

KP performed the experiments, analyzed the data, and wrote the manuscript. KA and KS-D contributed to the study design, performed the experiments, analyzed the data, and reviewed the manuscript. MR performed the experiments. AK was responsible for the study design, interpretation of findings, and writing of the manuscript. All the authors critically reviewed the content and approved the final version for publication.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Written informed consent was obtained from all Tartu University Hospital Blood Bank blood donors. The donors stated in the consent form that their blood could be used for scientific purposes. The blood samples were anonymized by the Tartu University Hospital Blood Bank. This study was approved by the local Ethics Review Committee on Human Research of the University of Tartu, Estonia (protocols 359/M-10). All methods were performed in accordance with the relevant guidelines and regulations.

ADDITIONAL INFORMATION

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