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Cannabidiol Improves Cognitive Impairment and Reverses Cortical Transcriptional Changes Induced by Ketamine, in Schizophrenia-Like Model in Rats

Ewa Kozela[1,2] · Martyna Krawczyk[3] · Tomasz Kos[3] · Ana Juknat[1,2] · Zvi Vogel[1,2] · Piotr Popik[3]

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Abstract

Cannabidiol (CBD), a non-psychotropic cannabinoid, demonstrates antipsychotic-like and procognitive activities in humans and in animal models of schizophrenia. The mechanisms of these beneficial effects of CBD are unknown. Here, we examined behavioral effects of CBD in a pharmacological model of schizophrenia-like cognitive deficits induced by repeated ketamine (KET) administration. In parallel, we assessed transcriptional changes behind CBD activities in the prefrontal cortex (PFC), the main brain area linked to schizophrenia-like pathologies. Male Sprague-Dawley rats were injected for 10 days with KET followed by 6 days of CBD. The cognitive performance was evaluated in the novel object recognition test followed by PFC dissections for next-generation sequencing (RNA-Seq) analysis and bioinformatics. We observed that KET-induced learning deficits were rescued by CBD (7.5 mg/kg). Similarly, CBD reversed transcriptional changes induced by KET. The majority of the genes affected by KET and KET-CBD were allocated to astroglial and microglial cells and associated with immune-like processes mediating synaptogenesis and neuronal plasticity. These genes include C1qc, C1qa, C1qb, C2, and C3 complement cascade elements, Irf8 factor and Gpr84, Gpr34, Cx3cr1, P2ry12, and P2ry6 receptors. The main pathway regulators predicted to be involved included TGFβ1 and IFNγ. In addition, CBD itself upregulated oxytocin mRNA in the PFC. The present data suggest that KET induces cognitive deficits and transcriptional changes in the PFC and that both effects are sensitive to a reversal by CBD treatment.

Keywords Schizophrenia · Cognitive impairment · Cannabidiol · Ketamine · Gene expression · Oxytocin

Introduction

Compelling evidence points to the involvement of the endocannabinoid system (ECB) and its modulators in the pathology of schizophrenia. The ECB comprises of endogenous ligands including N-arachidonoyl ethanolamide (anandamide, AEA, [1, 2]) and 2-arachidonoyl-glycerol (2-AG, [3]), G-protein coupled CB1 and CB2 receptors an enzymatic

Main findings:
1. Cannabidiol rescues learning deficits in schizophrenia-like conditions induced by ketamine administration.
2. Cannabidiol reverses transcriptional changes induced by ketamine in the rat prefrontal cortex.
3. Cannabidiol and ketamine treatments affect the transcription of genes allocated to astroglial and microglial cells and mediating neuronal plasticity.

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✉ Ewa Kozela
ewakozela@yahoo.com

[1] The Dr Miriam and Sheldon G. Adelson Center for the Biology of Addictive Diseases, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

[2] Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel

[3] Department of Behavioral Neuroscience & Drug Development, Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland

machinery controlling "on demand" synthesis and degradation of the endogenous ligands [4]. The ECB system and its role in schizophrenia spectrum of disorders were described subsequent to the identification of Δ9-tetrahydrocannabinol (Δ9-THC, THC), the main psychotomimetic constituent of *Cannabis* plant binding to the CB1 and CB2 receptors, as well as of cannabidiol (CBD), the main non-psychotomimetic phytocannabinoid. Interestingly, THC and CBD exert opposite subjective effects in humans. The exposure to THC may induce a transient psychotic-like state in healthy individuals, including hallucinations, delusions, and cognitive impairment [5–7], may augment psychotic episodes, and may provoke relapses in schizophrenic patients [8–10]. On the contrary, CBD as early as in 1970s was shown to ameliorate schizophrenia-like symptoms in THC-intoxicated individuals, including healthy volunteers [11–14], revealing its antipsychotic activity. Moreover, it was observed that increased CBD content in *Cannabis* preparations has protective effects against THC-induced hallucinations or delusions [15–17]. In a case study, CBD reduced schizophrenia symptoms being superior to haloperidol, a typical antipsychotic drug with severe side effects [18] and decreased psychotic symptoms in open-label study in Parkinsons patients [19]. Double-blind controlled study in schizophrenia patients showed similar efficacy of CBD compared with amisulpride, one of the most effective antipsychotics in current use, in diminishing the disease symptoms. The same study revealed preferential side effect profile of CBD [20]. More recently, however, Boggs et al. [21] reported that CBD did not improve cognitive impairments associated with schizophrenia in stable antipsychotic-treated outpatients.

In agreement with these observations in humans, CBD was shown to improve performance in various schizophrenia-like models in animals. CBD restored deficits in prepulse inhibition of startle response (PPI), a measure of impaired sensorimotor gating characteristic for schizophrenia. The PPI improvement was reported in genetic [22] and pharmacologic schizophrenia-like approaches including inhibition of glutamatergic transmission/NMDA receptors [23, 24] or intra-accumbens amphetamine [25]. Moreover, CBD diminished stereotyped behavior and hyperlocomotion induced by psychotomimetic drugs, the paradigms serving as behavioral equivalents of "positive" schizophrenia symptoms [23, 26, 27].

Interestingly, CBD has also been suggested to improve various cognitive deficits including spatial learning and memory, recognition and associative learning (reviewed by [28]). However, most of the data originate from animal models of neurodegenerative diseases, while data from animal models of schizophrenia are limited. Only recently, the group of Guimaraes showed that CBD improves cognitive performance in the novel object recognition (NOR) test in mice, disturbed by NMDA receptor inhibition (with MK-801) [24]. The mechanisms of the antipsychotic and possibly procognitive effects of CBD are unknown.

Here, we examined the effects of CBD in ketamine (KET)-induced schizophrenia-like cognitive impairment conditions. Sub-chronic, intermittent injections of KET, an NMDA receptor antagonist, at low, sub-anesthetic doses have been previously shown to produce a pattern of behavioral, neurochemical, metabolic, and transcriptional changes in rodents that reliably mirror those observed in schizophrenic brain [29–31]. To get an insight into the mechanisms of CBD and KET activities, we applied high-throughput transcription screening tools (next-generation sequencing [RNA-Seq] followed by bioinformatics) to reveal the transcriptional changes induced by both drugs in the prefrontal cortex (PFC). The PFC is considered the main brain region involved in the etiology of schizophrenia with various cellular, neurochemical, and functional changes suggested to contribute to the appearance of positive, negative, and cognitive symptoms [32].

We report here that KET administration induces cognitive impairment and affects the transcription of a number of genes in the PFC of the tested rats and that these changes are rescued by the CBD treatment. The transcriptional activity of KET and CBD involves immune-like genes and pathways, suggesting a dominant involvement of glial component (microglia and astroglia) in the beneficial, procognitive, and antipsychotic-like effects of CBD.

Materials and Methods

Animals

The experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee for Animal Experiments, Institute of Pharmacology, Krakow, Poland. Male Sprague–Dawley rats (Charles River, Germany) weighing ~ 250 g at the arrival were housed in the standard laboratory cages, under standard colony A/C controlled conditions: room temperature 21 ± 2 °C, humidity (40–50%), 12-h light/dark cycle (lights on: 06:00) with *ad libitum* access to food and water. Rats were allowed to acclimatize for at least 7 days before the start of the experimental procedure. During this week animals were handled for at least three times. Behavioral testing was carried out during the light phase of the light/dark cycle.

Behavioral Procedures

Rats were transferred to the experimental room for acclimation at least 1 h before the start of the experiment. Rats were tested in a dimly lit (25 lx) "open field" apparatus made of a dull gray plastic (66 × 56 × 30 cm). The floor of the apparatus was cleaned and dried after each measurement.

Procedure consisted of (a) the habituation to the arena (without any objects) for 5 min, 24 h before the novel object recognition (NOR) test, and (b) the NOR test session comprised of two trials separated by an inter trial interval (ITI) of 1 h. During the first trial (T1, familiarization), two identical objects (A1 and A2) were presented in the opposite corners, approximately 10 cm from the walls of the open field. In the second trial (T2, recognition), one of the objects was replaced by a novel one (A = familiar and B = novel). Both trials lasted 3 min. Animals were handed back to their home cages after T1. The objects used in the study were glass beakers filled with gravel and plastic bottles filled with sand. The heights of the objects were comparable (~ 12 cm), and the objects were heavy enough to unable their displacement by the animals. The sequence of presentations and the location of the objects was randomly assigned to each rat. The animals explored the objects by looking, licking, or touching the object while sniffing, but not when leaning against, standing or sitting on the object. Any rat spending less than 5 s exploring the two objects within 3 min of T1 or T2 was eliminated from the study. Exploration time of the objects and the distance traveled were measured using the *Any-maze*® video tracking system [33]. Based on exploration time (*E*) of two objects during T2, discrimination index (DI) was calculated according to the formula: DI = (EB − EA)/(EA + AB).

Experimental Design for Acute Experiment

The pilot/initial experiment was carried out to establish whether, and at which dose CBD can effectively reverse KET-induced impairment of learning (object discrimination) in the NOR test. In this experiment, KET was administered at the dose of 20 mg/kg, intraperitoneally (i.p.), 45 min before familiarization phase (T1) of NOR test. The CBD was administrated i.p. 30 min before KET injection.

Experimental Design for Sub-chronic Experiment

Scheme 1 illustrates the procedure of sub-chronic experiment. KET was injected i.p. at the dose of 30 mg/kg once daily (*o.d.*) for 10 consecutive days. Then, the rats underwent a washout period of 6 days during which they did not receive any injections. After the washout period, the NOR procedure started. Two NOR measurements were performed as follows: the first NOR measurement (NOR I) was conducted 6 days after the KET washout period, on day 17. NOR I was followed by 6 days of CBD i.p. injections of 7.5 mg/kg, twice daily (*b.i.d.*). Then, the second NOR measurement (NOR II) was carried out. The first and the last dose of CBD was administrated i.p. 1 h before familiarization phase (T1) of both NOR I and NOR II measurements. The two NOR tests were performed to evaluate if there is any change in the CBD effect following repeated administration of the drug. Different "novel objects" were used in NOR I and NOR II tests. The KET administration paradigm used here has been shown by us previously to effectively induce cognitive impairments in rats [31].

Drugs

KET (115.34 mg/ml of an aqueous solution, *Vetoquinol Biowet*, Gorzow Wielkopolski, Poland) was diluted in distilled water to the desired concentrations. CBD (*THC-Pharm Gmbh*, Frankfurt/Main, Germany) was dissolved in 10% aqueous solution of mixture of 1:1 ethanol with Kolliphor EL (Sigma-Aldrich, Poznan, Poland). All drugs were administered i.p. in the volume of 1 ml/kg of body weight.

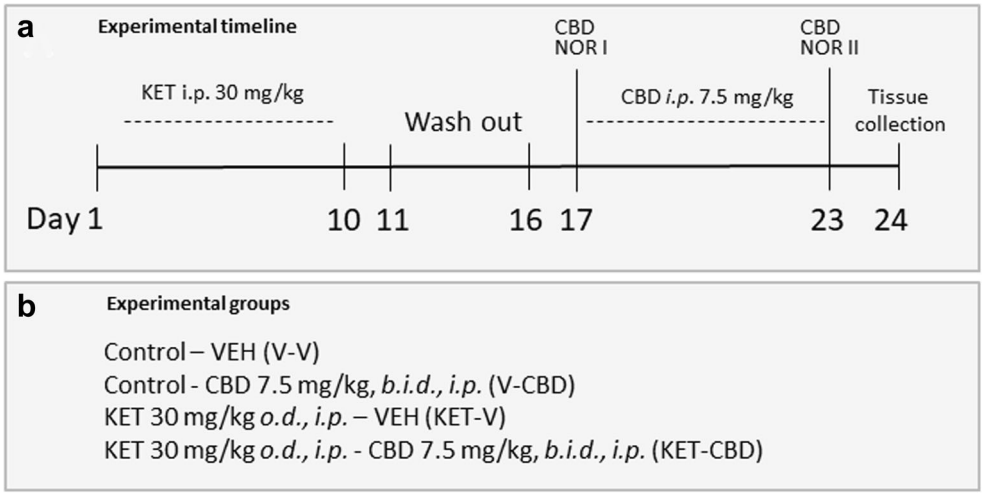
Tissue Collection and RNA Isolation

Twenty-four hours following the last CBD injection and NOR II measurement, the rats were anesthetized i.p. with 1 ml/kg of *Morbital* (*Biowet*, Puławy, Poland, containing 133.3 mg/ml of pentobarbital sodium and 26.7 mg/ml pentobarbital) and transcardially perfused with PBS. Subsequently, the PFCs were rapidly dissected, distributed into separate Eppendorf tubes, submersed in the *RNALater* reagent (*Thermo Fisher Scientific*, Waltham, MA, USA) and stored for further analysis.

Total RNAs from the collected PFC tissues were isolated using *QIAzol* reagent (*Qiagen*, Redwood City, CA, USA) and purified on silicon columns with on-column DNase digestion using *RNeasy Micro* kit (*Qiagen,* Redwood City, CA, USA). The concentration and purity of the RNA samples were measured using *NanoDrop One* spectrophotometer (*Thermo Fisher Scientific*). All the RNA samples submitted for further analysis were determined to have 260/280 and 260/230 values ≥ 1.9 and ≥ 2.0, respectively. The RNA integrity (RIN) was assessed using *The Agilent 2100 Tapestation*, and the average RIN value was 6.5 (range 6–7.2). Based on the RNA quality and purity parameters, a total of 28 samples were chosen and submitted for further analysis, with the following group sizes: control, vehicles only treated (V-V) $n = 6$, CBD-only treated (V-CBD) $n = 6$, KET-only treated (KET-V) $n = 8$, and KET+ CBD (KET-CBD) $n = 8$.

Library Preparation, RNA-Sequencing, and Bioinformatics Analysis

The RNA samples were submitted for the high-throughput RNA sequencing (RNA-Seq) analysis of the PFC whole transcriptomes using Next Generation Sequencing platform available at the Israel National Center for Personalized Medicine (INCPM) of the Weizmann Institute of Science, Rehovot, Israel. Sequencing libraries were prepared using INCPM in-house libraries. Single read reads were sequenced



Scheme 1 Study design (**a**) and experimental groups (**b**)

in one lane of the *Illumina NextSeq SR-75*. The output was ~ 19 million reads per sample.

Bioinformatics analysis included quality assurance (QA) and mapping of the reads to the genome using *TopHat* (*ver.* 2.0.10). Poly-A/T stretches and *Illumina* adapters were trimmed from the reads using *cutadapt* [34]. Resulting reads shorter than 10 bp were discarded. Reads were mapped to the *Rattus norvegicus* (*ver.* Rnor_6.0) reference genome using STAR [35], supplied with gene annotations downloaded from *Ensembl* genome browser www.ensembl.org (with *EndToEnd* option and "*outFilterMismatchNoverLmax*" set to 0.04). The number of reads for each gene in each sample (i.e., expression levels) was quantified using *HTSeq-count ver.* 0.6.1p1 [36], using the above gene transfer format (gtf). Data normalization, identification, and analysis of differentially expressed genes were performed using the *DESeq2 ver.* 1.6.3 package [37] with the *betaPrior*, *cooksCutoff*, and independent filtering parameters set to False. Raw *p* values were adjusted for multiple testing using the procedure of [38] (aka False Discovery Rate [FDR]) and are provided as "p.adj" values throughout the manuscript.

Pathway Analysis

Pathway and global functional analyses were performed using the *Ingenuity Pathway Analysis* (IPA®, *Qiagen,* www.qiagen.com/ingenuity).

qPCR Analysis

Selected gene products among those found by RNA-Seq analysis to be significantly affected by either KET or CBD or a combination of both were validated by quantitative real-time PCR (qPCR) method as described previously [39, 40], with a few modifications. Thus, 1 μg of total RNA from each rat PFC sample was reverse transcribed into cDNAs using *QuantiTect Reverse Transcription Kit* (Qiagen) that included genomic DNA removal. The cDNA of each chosen gene was amplified with a pair of specific primers designed using online *IDT PrimerQuest* tool (*Integrated DNA Technologies*, Skokie, IL, USA) and synthesized by *Hy-labs* (Rehovot, Israel). The primers used are listed in the online recourses Table S1. Cyclophilin A (Peptidylprolyl isomerase A a.k.a. *Ppia*) gene product was used for normalization as suggested by previous reports [41] and confirmed by us to be not affected by any of the experimental treatments using both, RNA-Seq and qPCR methods. The results of the qPCR analyses are presented as relative mRNA expression values (ratios); i.e., mRNA of the gene of interest was divided by the mRNA of *Ppia* housekeeping gene.

Statistics

Data were analyzed using *IBM SPSS ver. 24* and *GraphPad Prism 7* software with one-way (NOR test in acute treatment experiment) or mixed-design (NOR tests in subchronic treatment as well as qPCR experiments) ANOVAs followed by Sidak post hoc test. All the data were first subjected to Shapiro-Wilk test to examine if they stand to normal distribution criteria. Data that did not meet the normal distribution criteria were log-transformed and then subjected for statistics [42]. A value of $p < 0.05$ was deemed significant.

Results

Acute and Subchronic Treatment with CBD Prevents KET-Induced Disruption in the NOR Test

KET administration was previously shown by us to effectively impair cognitive performance in NOR test in rats [31]. Here,

we evaluate if single CBD administration at various doses affects the KET-induced cognitive impairment.

Figure 1 demonstrates that acute administration of KET (20 mg/kg, i.p., 45 min before T1 familiarization phase) induced cognitive deficit in the NOR test and that CBD given 30 min before KET injection prevented this cognitive deficit ($F(6,56) = 24.25$; $p < 0.001$). The effects of CBD were dose-related, specifically the doses of 7.5 and 30 mg/kg (but not 1.875, 3.75, and 15 mg/kg) normalized the KET-induced learning deficit. The dose of 7.5 mg/kg CBD, the lowest most effective dose in the acute trial, was chosen for the subchronic drug treatment experiments.

In the next step, we examined if CBD administration reverses the cognitive impairment following prolonged KET exposure in rats. Figure 2 demonstrates that 10-day treatment with 30 mg/kg of KET resulted in significant learning impairment, which in the same rats was observed both 7 (NOR I) and 13 days (NOR II) following the last KET dose. As tested 7 days past last KET dose (NOR I), the initial single dose of 7.5 mg/kg of CBD reversed KET-induced learning deficit. A similar effect was observed in rats treated with CBD twice daily for 6 days and tested 13 days after the last KET dose (NOR II), suggesting that no tolerance developed to the procognitive effects of CBD under these experimental conditions.

RNA-Seq Analysis

To gain the mechanistic insight into the behavioral effects, we applied the RNA-Seq method to reveal transcriptional changes mediating the beneficial effects of CBD in the KET-induced model of schizophrenia symptoms. We chose the PFC tissue for RNA-Seq analysis as the abnormalities in the PFC

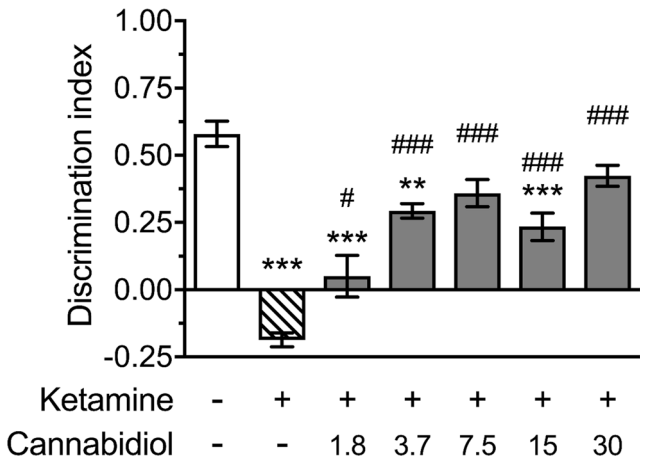


Fig. 1 Acute administration of KET (20 mg/kg, i.p., 45 min before T1 familiarization phase) induced cognitive deficit in the NOR test, which was prevented by pretreatment with CBD given 30 min before KET injection. Data are presented as discrimination index values, means ± SEM. *Symbols*: $^{**}p < 0.01$; $^{***}p < 0.001$ *vs* vehicle; $\#p < 0.05$; $\#\#\#p < 0.001$ *vs* KET. The N values for vehicle, KET, CBD 1.875, 3.75, 7.5, 15, and CBD 30 mg/kg were 7, 8, 9, 10, 10, 9, and 10 rats, respectively

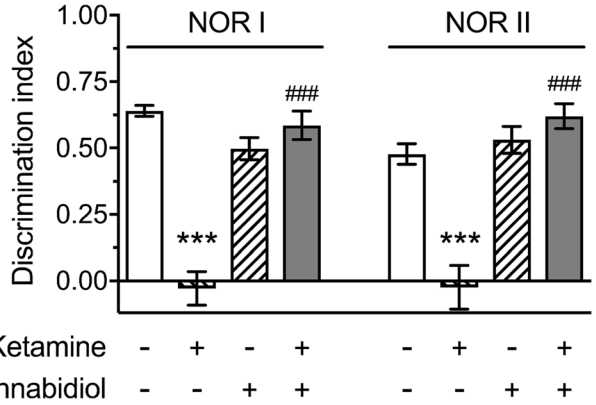


Fig. 2 Both, an acute (NOR I) and six-day CBD (7.5 mg/kg) administration (NOR II) improved KET-induced disruption of NOR performance. KET was administered for 10 days at 30 mg/kg/day. NOR I was performed 7 days after the last KET injection and NOR II was performed 13 days after the last KET dose. Data are presented as means ± SEM. Two-way mixed design ANOVA with NOR measurement as repeated factor and the treatment as between subjects factor demonstrated insignificant effects of NOR test number ($F(1,33) = 0.359$), the significant effects of CBD treatment ($F(3,33) = 61.561$; $p < 0.001$), and insignificant interaction ($F(3,33) = 1.522$). *Symbols*: $^{***}p < 0.001$ *vs* vehicle (V-V); $\#\#\#p < 0.001$ *vs* KET-V. The N was 9 for all groups except for the KET-V group where $N = 10$. *Symbols*: $^{***}p < 0.001$ *vs* V-V; $\#\#\#p < 0.001$ *vs* KET-V

function are widely linked to the symptomatology of schizophrenia and are mimicked in pharmacological models, including KET administration [31, 32].

Twenty-four hours following the last CBD injection and NOR II test, the PFC tissues of the examined rats were dissected. The total RNA was isolated and submitted for RNA-Seq analysis of the gene expression followed by bioinformatics analysis and qPCR validation of selected gene targets.

At the first stage of the analysis, the normalized counts obtained during the RNA-Seq running were ascribed the medians and the fold changes were calculated. The thresholds applied for differential expression analysis were as follows: p.adj ≤ 0.1, log2FoldChange ≤ − 0.585 or ≥ 0.585 (i.e., absolute fold change [FC] ≤ 0.67 or ≥ 1.5) and a minimal count for a gene expression ≥ 10. The analysis based on such stringent criteria revealed restricted number of genes that were differentially expressed in the PFC tissues across all experimental groups. Specifically, four genes were significantly downregulated in KET-treated rats (KET-V) as compared to control, vehicle only (V-V) treated rats, while 14 genes were upregulated in KET-CBD-treated rats in comparison to KET-V animals. None of the genes were significantly affected (did not meet the above criteria) in CBD-only-treated rats (CBD-V) as compared to V-V group.

The transcripts observed to be downregulated in the rat PFC by KET-V treatment included products of complement C1q C Chain gene (*C1qc* mRNA, FC = 0.6), NCK-associated protein 1 like gene (*Nckap1l* mRNA, FC = 0.59), cytohesin 4

(*Cyth4* mRNA, FC = 0.56), and G protein-coupled receptor 84 (*Gpr84* mRNA, FC = 0.42) (Table 1).

The transcripts found to be affected by KET-CBD treatment as compared to KET-V subjects were all upregulated (Table 1) and included oxytocin gene (*Oxt* mRNA, FC = 17), complement C3 (*C3* mRNA, FC = 2.6), serine peptidase inhibitor Kunitz type 1 (*Spint1* mRNA, FC = 2.4), GPR84 (*Gpr84* mRNA, FC = 2.2), integrin subunit alpha D (*Itgad* mRNA, FC = 1.9), GPR34 (*Gpr34* mRNA, FC = 1.9), complement C2 (*C2* mRNA, FC = 1.9), C1qa gene (*C1qa* mRNA, FC = 1.8), interferon regulatory factor 8 (*Irf8* mRNA, FC = 1.8), Rn50_2_1408.1 (*Rn50_2_1408.1* mRNA, FC = 1.7), lysosomal protein transmembrane 6 (*Laptm5* mRNA, FC = 1.7), NCKAP1L (*Nckap1l* mRNA, FC = 1.7), CYTH4 (*Cyth4* mRNA, FC = 1.6), and C1qc (*C1qc* mRNA, FC = 1.6). It is important to note that the gene transcripts upregulated by CBD post-treatment in KET-treated rats include four gene products that were downregulated by KET alone, i.e., *C1qc*, *Nckap1l*, *Cyth4*, and *Gpr84*, as specified above. This suggests that CBD reverses KET-induced transcriptional changes in the PFC of the tested rats (Table 1).

Identification of Gene Networks and Pathways Affected by CBD in the PFC of KET-Treated Rats

The genes with their respective identifiers, p.adj and log2FC values, were uploaded into the IPA engine in order to identify gene-gene interactions, top upstream regulators, and the pathways that mediate the CBD effect in KET-treated, cognitively tested rats. The inclusion parameters for the genes uploaded on the IPA were set at log2FC between ≤ -0.585 and ≥ 0.585 (i.e., absolute FC ≤ 0.67 or ≥ 1.5) and p.adj ≤ 0.5 (corresponding to $p \leq 0.028$). Widening the inclusion parameters allowed enriched insight into gene interactions and pathways possibly involved. However, due to our sole interest in the effects of the treatments in the brain tissue (PFC), the IPA set up was narrowed down to "the CNS cells and structures, including astrocytes and endothelial cells" and to "immune cells and structures."

In the KET-V group, the IPA analysis indicated a significant enrichment in "Complement system" (p.adj = 0.000000146) and "Role of pattern recognition receptors in recognition of bacteria and viruses" (p.adj = 0.00003) top canonical pathways as compared to control, V-V group. Within "Top regulators" category, KET was found to inhibit IFNG cytokine and TGFB1 growth factor signaling (Table 2). In parallel, PSEN1 peptidase pathway was identified as potentially activated following KET treatment. These IPA assumptions were founded on the KET-induced inhibition of gene expression of the genes *C1qa*, *C1qb*, *C1qc*, *Mmp2*, *Ptpn6*, among others, as listed in Table 2.

"Complement system" and "Role of pattern recognition receptors in recognition of bacteria and viruses" pathways were also identified to be significantly affected in the KET-CBD group as compared to KET-treated rats (p.adj = 0.00000269 and p.adj = 0.0000609, respectively). However, in this case, the IPA analysis predicted potential activation of IFNG and TGFB1 by CBD in rats previously treated with KET (KET-CBD) (Table 2). Moreover, PSEN1 pathway was identified as inhibited, reversing the KET effect. Additionally, *L-dopa*-dependent pathway was predicted by the IPA to be down-regulated (Table 2). These findings further suggest that

Table 1 Genes significantly affected by KET-V and/or by KET-CBD treatments in the rat PFCs as revealed by RNA-seq analysis (p.adj ≤ 0.1, FC ≤ 0.67 or ≥ 1.5). ns non-significant

Gene name	Ensemble gtf	KET-V vs V-V		KET-CBD vs KET-V	
		Fold change	p.adj	Fold change	p.adj
Downregulated in KET-V and reversed in KET-CBD					
C1qc	ENSRNOG00000012804	0.6	0.0061	1.6	0.0051
Nckap1l	ENSRNOG00000036829	0.59	0.019	1.7	0.0051
Cyth4	ENSRNOG00000007679	0.56	0.039	1.6	0.061
Gpr84	ENSRNOG00000036834	0.42	0.044	2.2	0.023
Upregulated in KET-CBD (vs KET-V)					
Oxt	ENSRNOG00000021225	–	ns	17	0.045
C3	ENSRNOG00000046834	–	ns	2.6	0.0051
Spint1	ENSRNOG00000012811	–	ns	2.4	0.0059
Itgad	ENSRNOG00000019728	–	ns	1.9	0.093
Gpr34	ENSRNOG00000039759	–	ns	1.9	0.035
C2	ENSRNOG00000051235	–	ns	1.9	0.052
C1qa	ENSRNOG00000012807	–	ns	1.8	0.023
Irf8	ENSRNOG00000017869	–	ns	1.8	0.0094
Rn50_2_1408.1	ENSRNOG00000051193	–	ns	1.7	0.045
Laptm5	ENSRNOG00000011054	–	ns	1.7	0.061

Table 2 The IPA-identified top upstream regulators affected by KET and by KET-CBD in the PFC of rats examined in the NOR tests

	KET-V vs V-V			KET-CBD vs KET-V		
Upstream regulator	Predicted activity	Activation *z*-score	Target molecules	Predicted activity	Activation *z*-score	Target molecules
TGFB1	↓	− 2.6	C1QA, C1QB, C1QC, ITGB2, MMP2, PTPN6, SELPLG, SPI1	↑	2.97	C1QA, C1QB, C1QC, CX3CR1, CYBB, FCGR3A/FCGR3B, ITGB2, MPZ, P2RY6, PTX3, SELPLG, SPI1
IFNG	↓	− 2.3	C1QA, C1QB,C 1QC, CSF1R, CTSC, CTSS, IRF8, ITGB2, MMP2, PTPN6, RAC2, TRAF6	↑	2.81	C1QA, C1QB, C1QC, CCL19, CSF1R, CTSS, CX3CR1, CYBB, FCGR3A/FCGR3B, IRF8, ITGB2, LAT2, P2RY6, PTX3, TLR7
PSEN1	↑	2.4	C1QA, C1QB, C1QC, CTSC, CTSS, SELPLG	↓	− 2.23	ARL11, C1QA, C1QB, C1QC, CTSS, SELPLG
L-dopa	–	–	–	↓	− 2.24	C1QA, CYTH4, LAPTM5, LAT2, P2RY6

CBD was able to reverse transcriptional changes and pathway activities induced by KET in the PFC of experimental rats, along with improving their KET-impaired cognitive performance.

The IPA Analysis of Gene-Gene Interactions

Figure 3 illustrates the IPA-assembled interactome (gene-gene interaction scheme). The network consists of (a) genes that were recognized by the IPA to be significantly affected by the KET-CBD treatment in comparison to KET-V subjects (FC ≥ 1.5 or ≤ 0.67 and p.adj < 0.5), (b) as well as of genes/top regulators/pathways possibly affected. The IPA analysis denoted IFNG, IL-2, and APP as the main pathway regulators involved based on the upregulated levels of *C1qa*, *C1qb*, *Cd84*, *Cx3cr1*, *Csf1r*, *Ctss*, *P2ry6* mRNA and downregulated *Ptx3* mRNA (for IFNG cytokine-dependent pathway), upregulated levels of *Parp14*, *Arl1*, *Csf1r* mRNA (for APP1-related pathway) and upregulated levels of *Card11*, *Fcgr3a*/*Fcgr3b*, *Laptm5*, *Cyth4* (for IL2-dependent pathway). Interestingly, the genes and top regulators found to be involved are mainly the mediators of immune responses including elements of complement cascade, signaling pathways and cytokines. This indicates a dominant involvement of local immunocompetent cells, i.e., microglia and astrocytes, in the KET-mediated changes as well as in the reversal of KET effects by CBD. Indeed, genes such as C1qa, C1qb, C1qc, Cxc3cr1, P2ry12, P2ry6, Gpr34, Gpr84, Ptx3, and Irf8 were reported previously to be either uniquely expressed by microglia or co-expressed on microglial cells and astrocytes [43, 44].

An additional IPA analysis restricted to "CNS structures and cells: neurons and astrocytes" was carried out in parallel to check if lesser immunocompetent context can reveal the involvement of other pathways. Nevertheless, the online recourses Figure S1 shows that again, the IPA linked the effects of CBD in KET-treated rats to IFNG, TNF, TGFB1, PSEN1, and L-dopa signaling.

Validation by qPCR

The level of several gene products, revealed by RNA-Seq analysis to be affected by the present experimental conditions, was validated by qPCR and with the use of cyclophylin A (*Ppia*) as a reference gene. Gene products selected for qPCR evaluation included *C1qc*, *C3*, *Gpr84*, and *Oxt* mRNAs. In addition, we examined the transcriptional activity of two additional gene products. Due to the high representation of microglia-related genes, we included allograft inflammatory factor 1 (*Aif1*, aka ionized calcium-binding adapter molecule 1 [*Iba1*]) mRNA, a common marker of microglial activity. These transcripts represented a variety of changes in our experimental conditions, allowing the accurate validation of the RNA-Seq results. Among non-affected genes, we studied the levels of *Zfp804a* mRNA, a schizophrenia risk gene [45].

Statistical analysis of the qPCR data was performed on "gene of interest/Ppia" ratios using "copy number per reaction" values provided by the qPCR analysis software (Rotor-Gene 6000, Qiagen). Only runnings with efficiency 0.9–1.1 were included. In addition, RNA expression levels were quantified using "the comparative cycle of threshold (Ct) method" (ΔΔCt; [46]) and expressed as averaged fold change vs control group (where mRNA expression is equal to 1). All these values are presented in the online recourses Table S2.

qPCR analysis showed that KET decreased the *C1qc* mRNA levels by 35% as compared to control rats (*C1qc*/*Ppia* ratio = 0.89 and 1.38, respectively; Fig. 4a). A value similar to the change observed using the RNA-Seq analysis (Table 1). Six-day CBD treatment following the 10 days of KET injections significantly increased the *C1qc* mRNA expression back to baseline (control) levels (*C1qc*/*Ppia* ratio = 1.52, by 59%; Fig. 4a), a change which corresponds well to the RNA-Seq-detected changes (Table 1). CBD given alone did not affect the levels of C1qc mRNA (*C1qc*/*Ppia* = 1.05; Fig. 4a). Two-way ANOVA revealed no effect of KET and no effect of CBD given alone although it showed significant KET and CBD interaction ($p < 0.01$; detailed ANOVA values for all

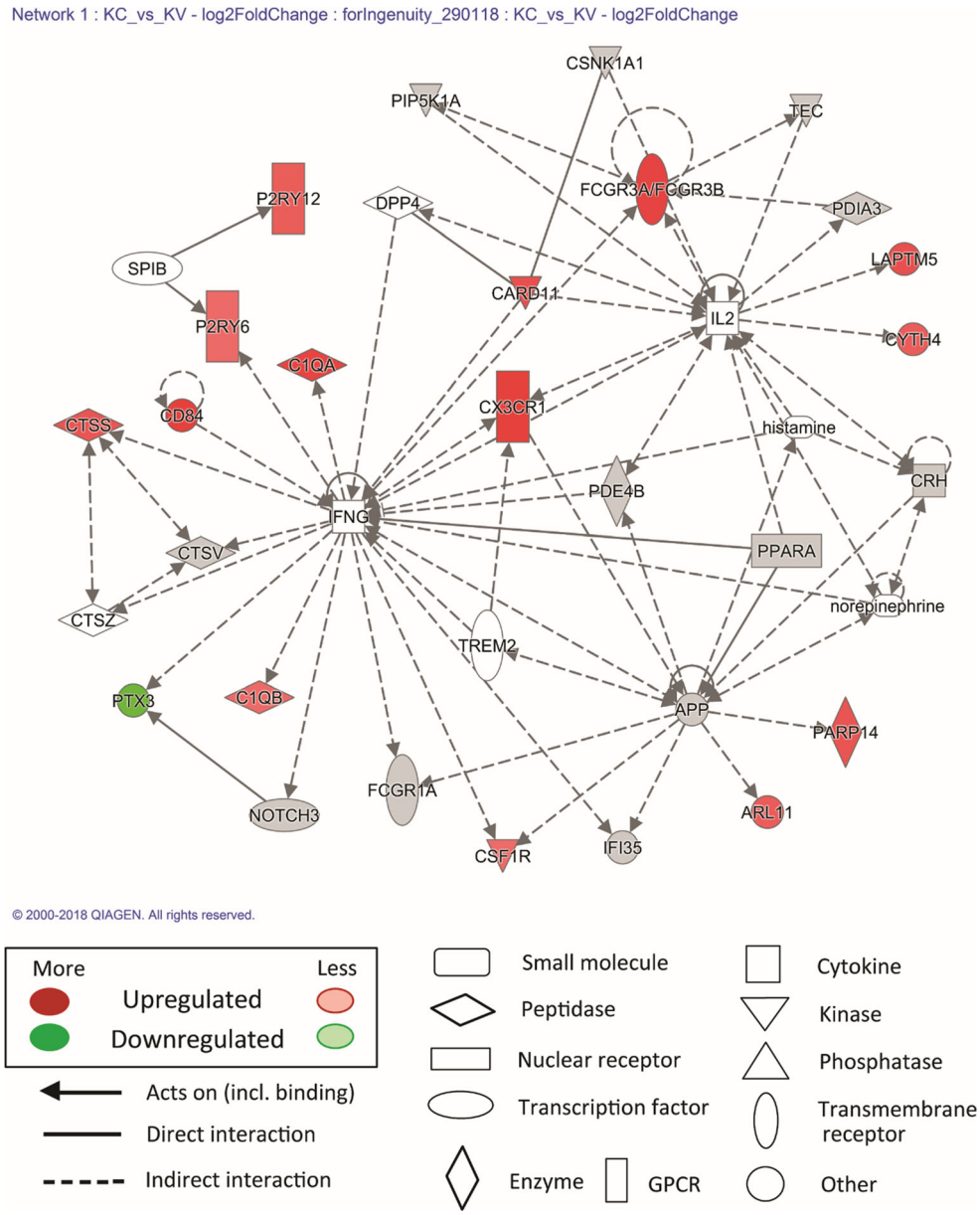


Fig. 3 The IPA-assembled interactome showing CBD effects in the PFC of KET-injected rats. The direction of modulation is color coded, with nodes in red indicating upregulation, and in green indicating downregulation by CBD (KET-CBD) *vs* KET only treated rats. The molecules in grey were shown in the literature (the IPA in-house database) to interact with the colored gene products. The legend of the figure provides an explanation of the node shapes

qPCR validated genes are shown in the online recourses Table S3), while Sidak's post hoc test confirmed significant difference between KET-treated and KET-CBD subjects ($p < 0.05$) for *C1qc* mRNA expression. mRNA fold changes calculated using ΔΔCt method were as follows: V-V = 1, KET-V = 0.6, KET-CBD = 1.1, and V-CBD = 0.8 (online recourses Table S2).

qPCR analysis revealed that KET decreased by 41% *C3* mRNA levels from *C3*/*Ppia* ratio = 0.29 in control group to 0.17 (KET-V). KET-CBD treatment increased the *C3* mRNA levels back to baseline levels (*C3*/*Ppia* = 0.41), an upregulation by 135% which is similar to the change observed in RNA-Seq analysis (Table 1). CBD given alone did not affect the C3 mRNA expression (*C3*/*Ppia* = 0.18). Two-way ANOVA was performed on log-transformed *C3*/*Ppia* mRNA ratios (Fig. 4b) and showed no effect of KET given alone, no effect of CBD given alone but revealed a significant interaction of both drugs ($p < 0.05$). Post hoc analysis did not show, however, further significant differences (Fig. 4b). For ΔΔCt-derived fold changes of C3 mRNA expression, see online recourses Table S2.

qPCR analysis showed that KET decreased *Gpr84* mRNA levels by 45%, i.e., from Gpr84/Ppia ratio = 1.16 in V-V control group to 0.64 in KET-V subjects (Fig. 4c), corresponding to a decrease observed in RNA-Seq analysis (Table 1). Again, KET-CBD treatment increased the *Gpr84* mRNA levels back to baseline levels (*Gpr84*/*Ppia* = 1.21), i.e., by 90%, corresponding to the change seen in RNA-Seq. Two-way ANOVA showed no KET effect and no CBD effect; however, it revealed a significant interaction between CBD

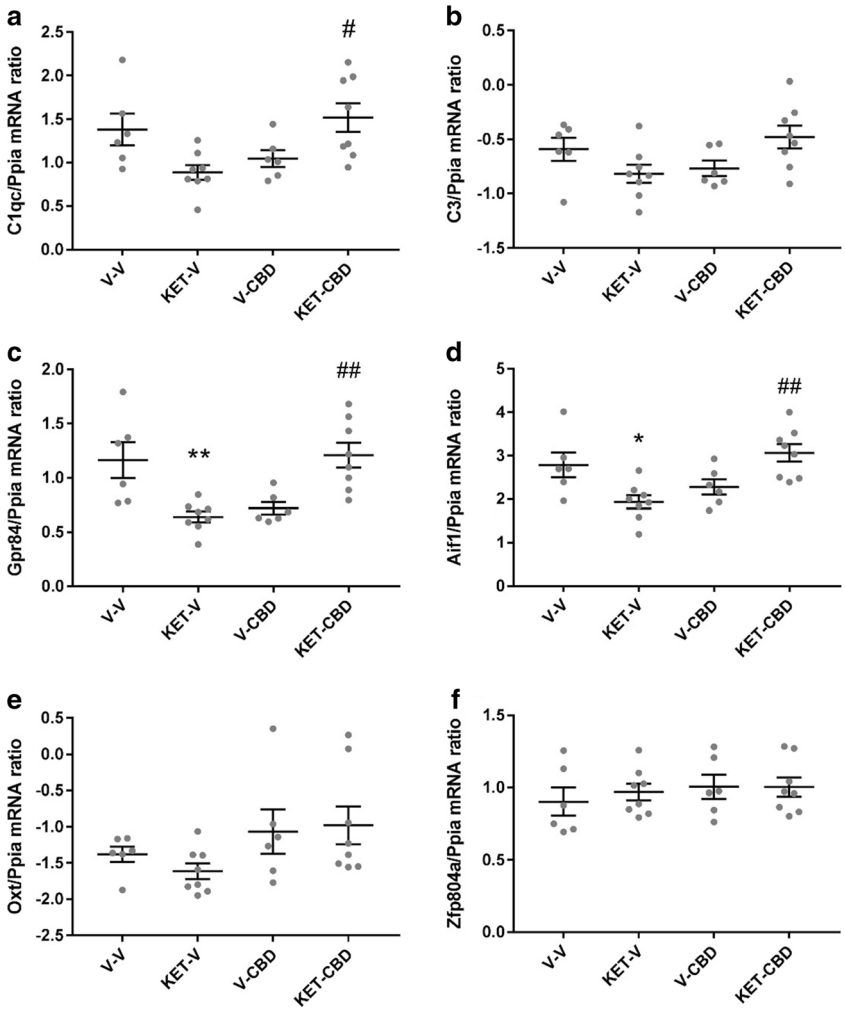


Fig. 4 qPCR analysis of selected mRNAs in the PFC of rats treated subchronically with KET followed by CBD (or vehicle) and examined in the NOR tests. The figure shows relative mRNA expression values calculated for all individual samples (open grey circles) together with the group averages ± SEM: **a** *C1qc*, **b** *C3*, **c** *Gpr84*, **d** *Aif1*, **e** *Oxt*, and **f** *Zfp804a* mRNA/*Ppia* ratios. The mRNA/*Ppia* ratios were subjected to two-way ANOVA followed by Sidak's post hoc test for multiple comparisons. $p < 0.05$ is considered significant. $^{*}p < 0.05$, $^{**}p < 0.01$ vs V-V, control group and $\#p < 0.05$, $\#\#p < 0.01$ vs KET-V. Number of subjects (*N*) were 6 in V-V and V-CBD, and 8 in KET-V and KET-CBD. Note that for C3 and Oxt read-outs, the data were log-transformed as the initial results showed non-normal distribution

and KET ($p < 0.0001$). CBD given alone non-significantly downregulated *Gpr84* mRNAs (*Gpr84*/*Ppia* = 0.72, $p = 0.055$; Fig. 4c)

qPCR analysis showed that KET-treatment lowers the expression of *Aif1* mRNA in the PFC of rats by 30%, from Aif1/Ppia = 2.78 to 1.94. CBD injections in KET-treated rats upregulated the *Aif1* mRNA (3.06), while CBD given alone had no effect (2.28). Two-way ANOVA showed no KET effect and no CBD effect but revealed significant KET and CBD interaction ($p < 0.001$; Fig. 4d).

Oxt mRNA was not significantly affected by KET (Oxt/Ppia = 0.05 in V-V and 0.03 in KET-V) as examined using qPCR. However, the *Oxt* mRNA levels were found to be increased by CBD in both, KET-CBD and V-CBD groups (*Oxt*/*Ppia* = 0.42 and 0.42, respectively). The log-transformed *Oxt*/*Ppia* values were subjected to two-way ANOVA. The analysis showed no significant interaction between KET and CBD as well as no effect of KET treatment alone but instead a significant effect of CBD alone ($p < 0.05$; Fig. 4e), altogether corresponding to RNA-Seq results.

With similarity to RNA-Seq, the *Zfp804a* mRNA was not affected by any of the experimental treatments when evaluated using qPCR (Fig. 4f).

Discussion

We report that CBD, a non-psychotomimetic cannabinoid, rescues the KET-induced learning impairments in an animal pharmacological model of schizophrenia symptoms. Next-generation sequencing followed by bioinformatics analysis revealed that CBD reversed KET-induced changes in gene transcription in the PFC area of the tested rats, the main brain structure associated with the schizophrenia symptoms. The repertoire of genes, which activity was affected by KET and by KET-CBD, indicates the predominant involvement of glial cells, microglia, and astroglia in the antipsychotic and procognitive effects of CBD described here.

Cognitive impairment is a core symptom underlying schizophrenia-spectrum of disorders. Despite years of

intensive research, current pharmacotherapies of this disorder target mainly the positive symptoms, having poor or no benefits for the negative and cognitive ones [47]. There is a mounting evidence indicating antipsychotic effects of CBD (reviewed by [48–50]). However, much less is known about the CBD effects on cognition. Studies in *Cannabis* users show improvement of episodic and recognition memory, verbal learning, and memory following CBD [51, 52] as well as no effects [53] (reviewed by [28]). Several preclinical studies showed improved working memory, object and social recognition, spatial learning and memory in several neurodegenerative models including the model of Alzheimer's disease [54–56], in inflammation [57–59] and in neurological deficits, following both, acute or chronic CBD treatment (reviewed by [28]).

The effects of CBD on cognition in psychiatric disorders have been barely addressed so far. Studies involving psychiatric patients showed minor procognitive effects of CBD either given acutely [60], chronically [50], or in combination with other antipsychotics [21]. In animals, acute CBD did not improve MK-801-induced social recognition memory deficits in rats [61] although it was protective against MK-801-impaired NOR in mice at high (60 mg/kg) doses given chronically [24]. In our hands, acute and subchronic administration of CBD appeared to have potent procognitive effects on NOR performance impaired by KET. Altogether, these findings suggest that the effect of CBD may be specific to the pathology and/or the cognitive domain tested, as well as to procedural differences contributing to the diverse outcomes (animal strain, administration protocol, and doses used). Moreover, as suggested by Boggs et al. [21], CBD may be effective in early but not in advanced stages of schizophrenia [21] (and refs within).

In the second part of our study, we aimed to decipher the molecular mechanisms involved in the antipsychotic and procognitive effects of CBD. In this regard, it is widely acknowledged that no single molecular event or individual gene can explain schizophrenia pathophysiology and that several genetic, developmental, environmental, and even nutritional factors may have a causative role [62]. We applied the next-generation sequencing method to assess the changes in total RNA expression in the PFCs isolated from KET- and CBD-treated rats. Our RNA-Seq results show that KET-induced changes in gene expression were reversed by CBD post-treatment. This pattern of gene regulation correlates with CBD rescue of KET-induced impairment of NOR performance, therefore possibly providing a mechanistic explanation of the behavioral effects. The RNA-Seq changes were validated with high correlation using qPCR method, further confirming the accuracy of the RNA-Seq results.

The majority of the genes affected by KET and/or KET-CBD treatments were associated with immune-like functions and were linked by bioinformatics-driven predictions to immune and immune-like processes. Top pathway regulators shown to be involved include TGFβ1 growth factor and IFNγ cytokine. Other genes affected include complement cascade elements such as C1qc, C1qa, C1qb, C2, C3, as well as Gpr84 and Gpr34 receptors, Irf8 transcription factor, Cx3cr1 chemokine receptor, P2ry12 and P2ry6 purinergic receptors. All of these genes were reported to be expressed predominantly by microglia, the resident brain immune cells, and were shown previously to be responsive to TGFβ signaling [43, 63]. Several of these genes were also shown to be induced in astrocytes, including Gpr84 [44], Cyth4 [64], Laptm5 [65], and TGFβ [66].

Although microglial cells are mainly known for their role in neuroinflammatory processes, mounting evidence shows that these cells actively shape normal neuronal wiring and brain functions. Microglia depletion or impaired function was shown to accelerate cognitive declines [67–69]. The mechanisms beyond these activities involve immune-like molecules such as the chemokine receptor Cx3CR1, a receptor mediating microglial migration and phagocytosis [70], complement C3/CR3 signaling [71], and purinergic signaling including P2ry12 [72], i.e., genes observed by us to be affected by our experimental treatments. Indeed, knockout of Cx3CR1 in mice resulted in impaired hippocampal synaptic structure and electrophysiological characteristics, reduced brain connectivity, and decreased social interest, the latter observed in neurodevelopmental disorders such as autism or schizophrenia [73]. Moreover, genetically impaired Cx3CR1 signaling has been linked to schizophrenia in humans [74, 75].

The complement cascade is a classic innate immune system that recognizes and eliminates invading pathogens, foreign antigens, and cellular debris. Upon activation, C1q protein binds to pathogen surface and initiates a sequence of proteolytic reactions including C3b protein recruitment and its binding to CR3 receptors on the phagocyte surface, resulting in pathogen elimination [66, 76]. The same complement cascade localized at neurons participates in synapse elimination (aka pruning) during postnatal development, in mature brain and during aging. Microglia are the only cells in the brain that express CR3, binding and eliminating weak or superfluous synapses (marked by C1q and C3) and leaving the strongest connections (for refs see [71]). Thus, aberrant regulation of the complement cascade in the brain results in behavioral and cognitive changes and is proposed as a shared etiological pathway in several neurological and neuropsychiatric diseases, including schizophrenia [77]. C1q knockout results in hyperconnected neural wiring, facilitated seizures, memory loss, and other cognitive deficits [78]. As C1q and C3 complement mRNAs were found by us to be downregulated by KET and rescued following CBD post-treatment, we assume that this pathway is involved in procognitive effects of CBD.

GPR84 receptors from the rhodopsin superfamily are expressed predominantly on microglia [43] and astrocytes

[44]. GPR84 knock out in APP/PS1 mice (a transgenic model of Alzheimer's disease) resulted in stronger β-amyloid toxicity and deeper cognitive decline [79]. Microglia-specific P2Y12, a Gi-coupled ADP receptor sensing the ATP gradients generated (e.g., by local CNS injury), mediates the microglial motility (aka chemotaxis) [80]. Irf8 is known as the main transcription co-factor in microglia maturation and survival, and its deletion results in decreased microglial density and function [81]. The activity of GPR84, P2y12, and Irf8 genes was found by us to be downregulated by KET, suggesting a severe decline in microglia function and/or number, and subsequent rescue by CBD post-treatment. Moreover, we observed a similar pattern of regulation for Aif1 mRNA levels, a common and reliable marker of microglial cells across species [82], confirming a major role of microglia in the KET and CBD effects in the PFC.

The bioinformatics analysis pointed to TGFβ as a primary superior regulator of the affected genes. Astrocytes were shown to be the main source of TGFβ, and *via* this pathway to induce neurons to massively express C1q, initiating synapse refinement [63]. Astrocytes play an essential role in brain physiology, including in neuronal circuit formation and synaptogenesis [83], and their disrupted function results in various neuropathologies, including schizophrenia. Developmentally impaired astroglial function is translated into defective astrocytic maturation, abnormal transcriptional activity, poor myelination, and early onset of schizophrenia-like behavioral phenotype [84], a pattern correlating with hypomyelination as observed in schizophrenic patients (refs within [84]. Cyth4 is another astrocyte located gene with neuroprotective role [85] that was observed to be affected by the present treatments. Cyth4 was recently suggested as a risk gene in schizophrenia [64].

IFNγ was pointed as another superior regulator of the transcriptional changes described here. Indeed, lack of IFNβ cytokine and IFNγ-dependent signaling resulted in spontaneous dopaminergic neurodegeneration in mice, impaired motor activity, and learning, while recombinant IFNβ or overexpression promoted neurite growth and branching indicating a protective role for IFNβ in neuronal homeostasis, motor and cognitive performance [86]. Protective role of type I interferon signaling has been suggested in autism and other neurodevelopmental diseases [87].

We would like to note that we limited the current discussion solely to the regulators revealed by us using described bioinformatics approaches and *in silico* predictions. Although these tools are widely acknowledged, they may not be exhaustive for the subject. Therefore, we cannot exclude that other factors might regulate in parallel the transcriptional changes described here.

Recent human, post-mortem global transcriptomics analyses strongly link schizophrenia to immune system, inflammatory-like processes and oxidative stress [45, 77, 87–89]. For example, B cells [45], certain variations of the major histocompatibility complex locus [47] and alleles of the C4a and C4b were shown to be highly associated with schizophrenia [77]. These data do not disregard anomalies in neuronal circuits in the etiology of psychotic disorders but rather suggest that distorted neurotransmission, such as in schizophrenia, might be secondary to dysregulated glial activity. This assumption may explain the limited efficacy of current pharmacotherapies of schizophrenia, exclusively targeting dopaminergic and serotoninergic neurotransmission. Indeed, the anti-inflammatory medications show beneficial effects as an adjunctive therapy in schizophrenia [90], further confirming the role of immune-like processes in this neuropathology. Moreover, neuroinflammatory processes were linked to impaired cognition in psychiatric disorders [91]. It seems then that treatments targeting immune responses (such as CBD) may result in cognitive improvements in schizophrenic patients.

Mounting evidence suggest that endocannabinoids and exogenous cannabinoid ligands affect schizophrenia-like pathologies *via* modulation of glial activity [92]. CBD has been frequently shown by us and by others to be a potent regulator of the function and transcriptional activity of microglia [39, 93–95] and of astrocytes (for review see [96]. Recently, Gomes and colleagues showed that CBD decreases microglia activation in MK-801-induced model of schizophrenia in mice [24], indicating that CBD modulation of microglial activation contributes to antipsychotic and procognitive properties of this cannabinoid in various models.

The mRNA of oxytocin neuropeptide was upregulated by CBD in control and in KET-treated rats. Early findings indicate that oxytocin is associated with broad cognitive processes [97], while recent observations suggest that its administration may benefit the pharmacotherapy of schizophrenia. Intranasal oxytocin administration not only reduced classic psychotic symptoms but also improved social cognition (facial emotion recognition) and other cognitive scores in schizophrenic patients (reviewed by [98]). Thus, the increased oxytocin mRNA levels observed by us may contribute to procognitive and/or antipsychotic effects of CBD described here.

Oxytocin was shown recently to be a potent modulator of endocannabinoid tone. Oxytocin improved social performance and reward in autism-like animal model *via* upregulating AEA levels in the nucleus accumbens [99]. Increased AEA levels accompanied CBD antipsychotic effects in schizophrenic patients [20] (reviewed by [48]). We have shown previously that CBD increases AEA in microglial cells [100]. This suggests that CBD may engage Oxt pathway to upregulate endocannabinoid tone, this way exerting antipsychotic and procognitive effects. This hypothesis needs further examination.

Interestingly, we previously showed that chronic THC administration led to decreased *Oxt* mRNA and protein

expression in NAcc and ventral tegmental areas [101], opposite to the CBD effect described here. Such opposite activities of these cannabinoids on oxytocin levels may contribute to their antagonizing effects on behavior, including on schizophrenia-like symptoms [21, 102].

In summary, our results show that subchronic administration of KET induces cognitive impairment in rats which is accompanied by downregulated transcriptional activity of various microglial/astrogial genes involved in synaptogenesis. Both, KET impaired learning and transcriptional glial hypofunction are reversed by CBD post-treatment. Our results suggest that CBD may efficiently restore proper neuronal and cognitive functions in schizophrenia-like conditions, possibly *via* glia-mediated rescue of synaptic plasticity.

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Author Contributions T.K., M.K., and P.P. designed, performed, and analyzed the behavioral experiments. E.K. performed the mRNA extractions, the qPCR analysis of gene expression, the IPA analysis and wrote the article. E.K., T.K., M.K., A.J., Z.V., and P.P. contributed to discussions of article content, and to reviewing and editing the manuscript before submission.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interests.

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